[5] Fast, Facile, Hypersensitive Assays for Ribonucleolytic Activity

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Assays for Ribonucleolytic Activity

A sensitive and convenient assay for catalytic activity is an essential tool for studying catalysis. Because of the seminal role of bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) in the history of enzymology,[1] assays for ribonucleolytic activity have existed for decades. The recent discovery of ribonucleases[2] and ribozymes[3] with important biological functions, but low ribonucleolytic activity, has spurred the development of assays with high sensitivity.

Early work on the kinetics of catalysis by RNase A used substrates that were either ill-defined heterogeneous strands of RNA (e.g., yeast RNA in the "Kunitz" assay[4]) or nucleoside 2′,3′-cyclic phosphodiesters,[5] which are the products rather than the substrates of the germinal transphosphorylation reaction.[6,7] One appropriate application of assays using RNA polymers is for the detection of ribonucleolytic activity in a complex mixture. For example, the release of methylene blue from yeast RNA provides a sensitive assay at 688 nm, a wavelength of light not absorbed by most biomolecules.[8] Alternatively, zymogram assays can detect as little as 1 pg (0.1 fmol) of impure RNase A. In a zymogram assay, a polymeric substrate is incorporated into a gel, and cleavage is visualized by staining for intact polymers after electrophoresis[9–11] or isoelectric focusing.[12] A zymogram blot is also effective.[13]

[1] R. T. Raines, *Chem. Rev.* **98,** 1045 (1998).
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Answering questions about enzymatic catalysis with chemical rigor requires the use of a well-defined substrate. Homopolymeric substrates such as poly(uridylic acid) [poly(U)] and poly(cytidylic acid) [poly(C)] are now readily available. Further, the advent of modern phosphoramidite chemistry enables the facile synthesis of any di-, tri-, or tetranucleotide substrate. Because ribonucleases do not catalyze DNA cleavage, the synthesis of RNA/DNA chimeras extends further the horizon of possible analyses.[14,15]

Chromogenic substrates facilitate assays of ribonucleolytic activity. Uridine 3′-(5-bromo-4-chloroindol-3-yl)phosphate (U-3′-BCIP) is a substrate for RNase A.[16,17] The 5-bromo-4-chloroindol-3-ol product dimerizes rapidly in air to form a blue pigment. This substrate is analogous to 5-bromo-4-chloroindol-3-yl-galactose (X-Gal), a common substrate for β-galactosidase. Other chromogenic substrates rely on the production of yellow phenolates from the cleavage of a uridine 3′-arylphosphates.[18–20]

Fluorogenic substrates for fast, facile, and hypersensitive assays of ribonucleolytic activity are now known.[21–23] Fluorogenic substrates provides the basis for extremely sensitive assays for ribonucleolytic activity. 5′-*O*-[4-(2,4-Dinitrophenylamino)butyl]phosphoryluridylyl-(3′→5′)-2′-deoxyadenosine 3′-{*N*-[(2-aminobenzoyl)aminoprop-3-yl]phosphate} consists of a fluorophore (*o*-aminobenzoic acid) linked via Up(dA) to a quencher (2,4-dinitroaniline).[21] Cleavage of the phosphodiester bond in the Up(dA) linker results in a 60-fold increase in fluorescence, enabling the detection of a 50 f*M* concentration of RNase A. Substrates for even more sensitive assays have been reported and are available commercially. We describe herein the properties of these new fluorogenic substrates and associated protocols for assaying ribonucleolytic activity. We also describe applications for these substrates.

Design and Synthesis of Fluorogenic Substrates

The fluorogenic substrates were designed as RNA/DNA chimeras (Table I). Each substrate contains only one nucleotide with a ribose moiety, which defines a single cleavable site in the substrate. Such chimeric substrates are superior to polymeric or oligomeric RNA substrates for enzymological analysis because the

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[15] B. R. Kelemen and R. T. Raines, *Biochemistry* **38,** 5302 (1999).
[16] P. L. Wolf, J. P. Horwitz, J. Freisler, J. Vazquez, and E. Von der Muehll, *Experientia* **24,** 1290 (1968).
[17] M. R. Witmer, C. M. Falcomer, M. P. Weiner, M. S. Kay, T. P. Begley, B. Ganem, and H. A. Scheraga, *Nucleic Acids Res.* **19,** 1 (1991).
[18] A. M. Davis, A. C. Regan, and A. Williams, *Biochemistry* **27,** 9042 (1988).
[19] J. E. Thompson and R. T. Raines, *J. Am. Chem. Soc.* **116,** 5467 (1994).
[20] S. B. delCardayré, M. Ribó, E. M. Yokel, D. J. Quirk, W. J. Rutter, and R. T. Raines, *Prot. Eng.* **8,** 261 (1995).

TABLE I
OPTIMIZATION OF FLUOROGENIC SUBSTRATES FOR CLEAVAGE BY RIBONUCLEASE A[a]

Substrates	k_{cat}/K_m^b ($10^7\ M^{-1}s^{-1}$)	$F_{max}/F_0{}^c$	Sensitivity[d] ($10^8\ M^{-1}s^{-1}$)
6-FAM~rUdA~6-TAMRA	2.5 ± 0.3	15 ± 2	3.8 ± 0.7
6-FAM~dArU(dA)$_2$~6-TAMRA	3.6 ± 0.4	180 ± 10	65 ± 8
6-FAM~(dA)$_2$rU(dA)$_3$~6-TAMRA	4.7 ± 0.6	26 ± 3	12 ± 2
6-FAM~(dA)$_3$rU(dA)$_4$~6-TAMRA	4.8 ± 0.5	62 ± 2	30 ± 3

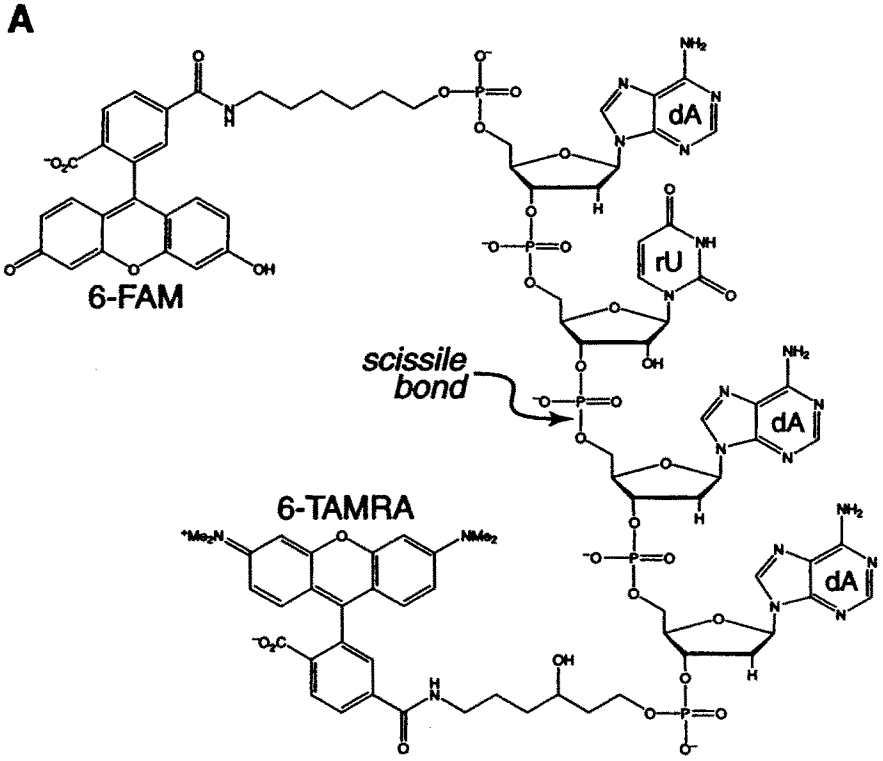
[a] Data are from Ref. 23.
[b] Values of k_{cat}/K_m were determined in 0.10 *M* MES–NaOH (pH 6.0) containing NaCl (0.10 *M*).
[c] F_{max}/F_0 is the ratio of fluorescence intensity of the product to that of the substrate.
[d] Sensitivity (S) is defined in Eq. (1).

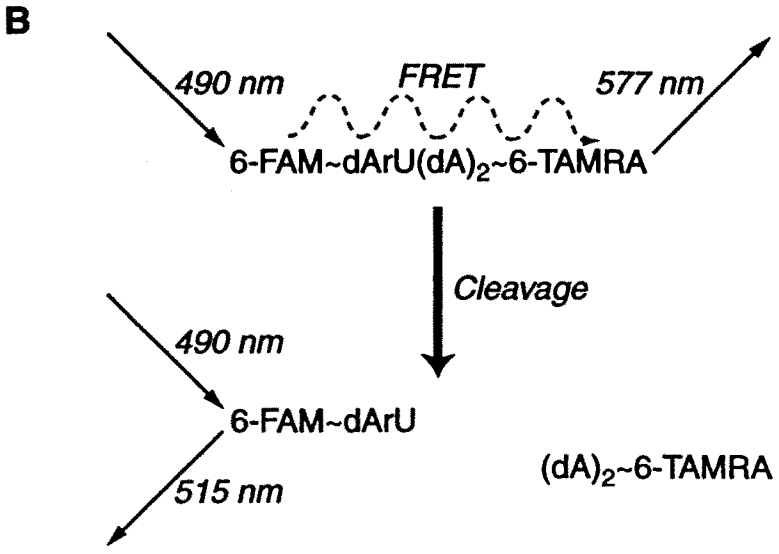
unique cleavable site provides for a homogeneous substrate. The sequences of the substrates were optimized for catalysis by RNase A. RNase A requires a pyrimidine residue 5′ to the scissile bond and prefers a purine residue 3′ to the scissile bond. The sole ribonucleoside residue has a uracil base. To minimize nonproductive binding and product inhibition, all of the deoxyribonucleotide residues are purines.

The assay of ribonucleolytic activity with these fluorogenic substrates is based on the fluorescence resonance energy transfer (FRET).[24] The substrates have a 5′ 6-carboxyfluorescein (6-FAM) label and a 3′ 6-carboxytetramethylrhodamine (6-TAMRA) label (Fig. 1). In an intact substrate, the fluorescence emission of the 6-FAM moiety at 515 nm is quenched by the proximal 6-TAMRA moiety (Figs. 1 and 2). Weak emission of the intact substrate is observed at 577 nm as a result of FRET from 6-FAM to 6-TAMRA. On substrate cleavage, the observed fluorescence emission of 6-FAM at 515 nm increases dramatically (Figs. 1 and 2). The net increase in fluorescence on substrate cleavage depends on the efficiency of FRET, and thus on the length of the substrate. A series of substrates with an incremental change in length substrate is defined as the product of F_{max}/F_0, the total increase in fluorescence intensity on substrate cleavage, and k_{cat}/K_M, the specificity constant of the enzyme, as in Eq. (1):

$$S = (F_{max}/F_0)(k_{cat}/K_m) \quad (1)$$

[21] O. Zelenko, U. Neumann, W. Brill, U. Pieles, H. E. Moser, and J. Hofsteenge, *Nucleic Acids Res.* **22,** 2731 (1994).
[22] D. A. James and G. A. Woolley, *Anal. Biochem.* **264,** 26 (1998).
[23] B. R. Kelemen, T. A. Klink, M. A. Behlke, S. R. Eubanks, P. A. Leland, and R. T. Raines, *Nucleic Acids Res.* **27,** 3696 (1999).
[24] J. R. Lakowicz, "Principles of Fluorescence Spectroscopy." Plenum, New York, 1999.


A
6-FAM
dA
rU
dA
dA
scissile
bond
6-TAMRA


B
490 nm
FRET
577 nm
6-FAM~dArU(dA)2~6-TAMRA
Cleavage
490 nm
6-FAM~dArU
515 nm
(dA)2~6-TAMRA

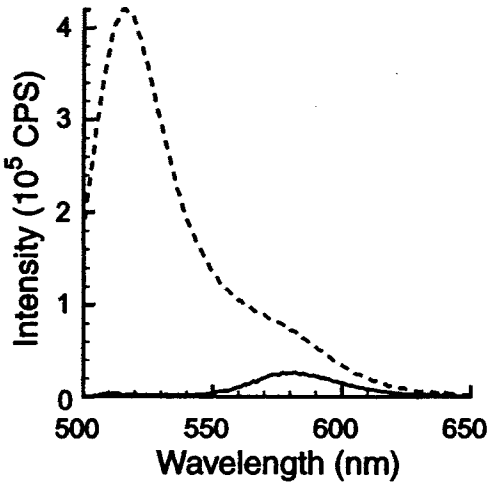


FIG. 2. Emission spectra of 6-FAM–dArU(dA)$_2$–6-TAMRA (solid line) and its cleavage product (dashed line) on excitation at 490 nm.[23] "CPS" refers to photon counts per second.

In Eq. (1), F_0 is the initial intensity before the reaction is initiated and F_{max} is the final fluorescence intensity after the reaction reaches completion. Thus, the value of S reports on the rate at which the fluorescence intensity changes at low concentrations of substrate. The value of S is unique for a particular enzyme and substrate and provides a useful means to compare assays of enzymatic activity.

The substrates can be synthesized by standard phosphoramidite chemistry on a conventional DNA/RNA synthesizer. [The most sensitive substrate for RNase A is available commercially from Integrated DNA Technologies (Coralville, IA; *http://www.idtdna.com*).] To minimize the background fluorescence from by-products, synthetic substrates are purified by high-performance liquid chromatography (HPLC). The concentration of the substrates can be estimated spectrophotometrically at 260 nm with extinction coefficients calculated from their sequences.[25]

[25] G. H. Beaven, E. R. Holiday, and E. A. Johnson, *in* "The Nucleic Acids" (E. Chargraff and J. N. Davidson, eds.), p. 493. Academic Press, New York, 1995.

FIG. 1. (A) Chemical structure of 6-FAM–dArU(dA)$_2$–6-TAMRA, the fluorogenic substrate for ribonucleases. The arrow indicates the scissile bond for ribonucleolytic cleavage. (B) Basis for the fluorescence assay of ribonucleolytic activity. The 6-FAM moiety is excited at 490 nm. In an intact substrate, the fluorescence emission of 6-FAM at 515 nm is quenched efficiently by the proximal 6-TAMRA moiety. Weak emission of the intact substrate is observed at 577 nm as a result of fluorescence energy transfer (FRET) from 6-FAM to 6-TAMRA. On substrate cleavage, the observed fluorescence emission of 6-FAM at 515 nm increases dramatically.

Preparation of Reagents

Pellets of synthetic substrates are dissolved in ribonuclease-free water[26] to a final concentration near 10 μM, dispensed into small aliquots, and stored at −20°. Fluorometric cuvettes (1.0 cm path length; 3.5 ml volume) are presoaked in nitric acid (10%, v/v) overnight to remove any residual ribonucleolytic activity. Quartz and glass cuvettes can be used interchangeably without any observable difference in assay results. Buffer [e.g., 0.10 M MES–NaOH (pH 6.0) containing NaCl (0.10 M)] is prepared with ribonuclease-free water and chemicals. Solutions should be handled with great care to minimize background ribonucleolytic activity from contamination with human ribonucleases.[27] Enzyme is diluted to a suitable concentration (e.g., 0.10–50 nM for RNase A) with the assay buffer.

Assay Procedures and Data Analysis

A typical assay for ribonucleolytic activity follows. Buffer (2.0 ml) is placed in a quartz or glass cuvette. A substrate (5.0 μl of a 10 μM solution) is added to the buffer with stirring to give a final concentration near 25 nM. The fluorescence emission is monitored at 515 nm, on excitation at 490 nm. The change in fluorescence intensity is monitored initially to check for unwanted ribonuclease contamination. The average fluorescence intensity at this point is designated as F_0, the initial fluorescence intensity. The reaction is initiated by adding enzyme (10 μl of a stock solution) with stirring.

The substrate concentration used in this assay is likely to be much lower than K_m. Thus, k_{cat}/K_m can be determined directly without applying Michaelis–Menten kinetics. Either an *exponential rise analysis* or *an initial velocity analysis* is used to calculate k_{cat}/K_m from the fluorescence change (Fig. 3). The two analyses typically produce equivalent results. If the reaction is fast enough to reach completion, V/K ($= (k_{cat}/K_m)[E]$) for the reaction is determined by an exponential analysis, fitting the observed fluorescence intensity to Eq. (2):

$$F = F_0 + (F_{max} - F_0)(1 - e^{-(V/K)t}) \tag{2}$$

In Eq. (2), F is the observed fluorescence intensity. Values of F_{max}, F_0, and V/K are determined by nonlinear regression analysis.

If a reaction is too slow to achieve completion, V/K is determined by an initial velocity analysis with Eq. (3):

$$V/K = \frac{(\Delta F/\Delta t)}{F_{max} - F_0} \tag{3}$$

[26] Y. H. Huang, P. Leblanc, V. Apostolou, B. Stewart, and R. B. Moreland, *Biotechniques* **19**, 656 (1995).

[27] R. Poulson, *in* "The Ribonucleic Acids" (P. R. Stewart and D. S. Letham, eds.), p. 333. Springer-Verlag, New York (1977).

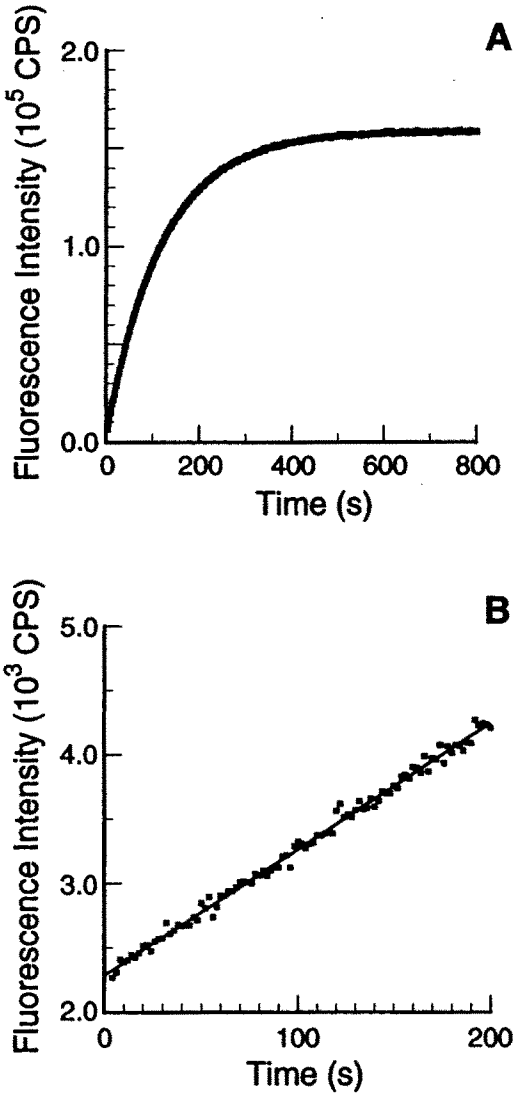


FIG. 3. Comparison of (A) exponential rise analysis and (B) initial velocity analysis of the cleavage of 6-FAM–dArU(dA)$_2$–6-TAMRA by ribonuclease A. Reactions were performed in 0.10 *M* MES–NaOH (pH 6.0) containing NaCl (0.10 *M*), 6-FAM–dArU(dA)$_2$–6-TAMRA (20 n*M*), and ribonuclease A [(A) 0.25 n*M*; (B) 2.5 p*M*]. "CPS" refers to photon counts per second.

In Eq. (3), $\Delta F/\Delta t$ is the slope determined from linear regression analysis of the fluorescence intensity change for an initial linear region. After the initial velocity is determined, F_{max} is measured by adding enough enzyme to complete the reaction. Because the fluorescence of the product can be sensitive to a change in pH or salt concentration, the enzyme used to complete the reaction should be in the assay buffer. The fluorescence intensity often decreases slowly after reaching a maximum, presumably because of the subsequent hydrolysis of the 2′,3′-cyclic phosphodiester product. The value of F_{max} can, however, be obtained from the observed maximum without introducing significant error. The value of k_{cat}/K_m can be calculated by dividing V/K by the concentration of enzyme in the reaction. Knowing the substrate concentration is not necessary for the determination of k_{cat}/K_m in either analysis.

Applications

Ribonuclease Contamination

Contaminating ribonucleolytic activity is the bane of everyone who works with RNA.[27,28] The fluorogenic substrates provide a most sensitive means to detect such contamination. For example, the activity arising from a 10 f*M* solution of human pancreatic ribonuclease, which is the human homolog of RNase A and a common laboratory contaminant, can be detected easily with a spectrofluorometric assay in 50 m*M* Bis–Tris-HCl buffer (pH 6.0).

Steady-State Kinetic Parameters

The hypersensitivity of assays based on fluorogenic substrates makes it possible to assay ribonucleolytic activity with low concentrations of substrates. Under these conditions, it is likely that [S] $\ll K_m$, so that the value of k_{cat}/K_m can be determined directly as described above. Two criteria can be applied to ensure that [S] $\ll K_m$. First, V/K will not change upon doubling the substrate concentration. If [S] is not far enough below K_m, then V/K will decrease. Second, fitting the fluorescence change data to Eq. (2) should not yield significant residual errors when the exponential rise analysis is used. Deviation of the data from Eq. (2) indicates that [S] is not far enough below K_m.

The hypersensitive assays also enable a quantitative description of catalysis by poor catalysts of RNA cleavage. For example, angiogenin is an RNase A homolog that promotes neovascularization. The k_{cat}/K_m values determined with RNase A and angiogenin are listed in Table II. Though k_{cat}/K_m values of angiogenin are 10^5-fold lower than those of RNase A, these values are determined without any

[28] S. L. Berger, *Methods Enzymol.* **152**, 227 (1987).

TABLE II
VALUES OF k_{cat}/K_m FOR CLEAVAGE OF FLUOROGENIC SUBSTRATES BY RIBONUCLEASE A AND ANGIOGENIN

Substrate	k_{cat}/K_m (10^7 $M^{-1}s^{-1}$)[a]	
	RNase A	Angiogenin
6-FAM–dArU(dA)$_2$–6-TAMRA	3.6 ± 0.4	0.000033 ± 0.000004
6-FAM–dArC(dA)$_2$–6-TAMRA	6.6 ± 0.6	0.000054 ± 0.000004

[a] Values of k_{cat}/K_m (± SE) were determined in 0.10 *M* MES–NaOH (pH 6.0) containing NaCl (0.10 *M*). Data are from Ref. 23.

complication. The assay conditions used to determine the ribonucleolytic activity of angiogenin were identical to those with RNase A, except that the concentration of angiogenin used in the reaction was higher (0.10–0.50 μM).

Substrate Specificity

Fluorogenic substrates that differ only in the nucleobase of the sole ribonucleotide residue can be used to determine the substrate specificity of a ribonuclease variant. For example, the T45G variant of RNase A catalyzes the efficient cleavage of poly(adenylic acid), whereas RNase A does not.[29] This change in substrate specificity is also manifested in the cleavage of fluorogenic substrates. Specifically, T45G RNase A cleaves 6-FAM–dArA(dA)$_2$–6-TAMRA 10^2-fold faster than does the wild-type enzyme (Table III). This finding is consistent with the observation that T45G RNase A is significantly more effective than the wild-type enzyme at cleaving heterogeneous RNA to completion.[30]

RNase T_1 from *Aspergillus oryzae* is not homologous to RNase A. This microbial ribonuclease is known to cleave preferentially the P–$O^{5'}$ bond after a guanosine residue.[31] As expected, a fluorogenic substrate that contains a guanosine ribonucleotide is cleaved at least 10^4-fold more quickly than are those that contain uridine, cytosine, or adenosine (Table III).

Inhibitor K_i Values

Fluorogenic substrates provide a simple method to determine the K_i value for competitive inhibition of ribonucleolytic activity. This method has been used to

[29] S. B. delCardayré and R. T. Raines, *Biochemistry* **33,** 6031 (1994).
[30] S. B. delCardayré and R. T. Raines, *Anal. Biochem.* **225,** 176 (1995).
[31] K. Takahashi, T. Uchida, and F. Egami, *Adv. Biophys.* **1,** 53 (1970).

TABLE III
VALUES OF k_{cat}/K_m FOR CLEAVAGE OF 6-FAM–dArXdAdA–6-TAMRA BY WILD-TYPE RIBONUCLEASE A, ITS T45G VARIANT, AND RIBONUCLEASE T_1 FROM *Aspergillus oryzae*

	k_{cat}/K_m (10^6 $M^{-1}s^{-1}$)[a]		
X	Wild-type RNase A[b]	T45G RNase A[b]	RNase T_1
U	36 ± 3	0.75 ± 0.08	<0.000001[c]
C	66 ± 6	3.3 ± 0.3	<0.000001[c]
A	0.000018 ± 0.000002	0.0023 ± 0.0002	0.00012 ± 0.00001
G	<0.0000001[c]	<0.0000005[c]	1.3 ± 0.1

[a] Values of k_{cat}/K_m (± SE) were determined in 0.10 *M* MES–NaOH (pH 6.0) containing NaCl (0.10 *M*).
[b] Data from Ref. 37.
[c] The activity is below the detection limit.

determine K_i values for inhibition of RNase A by substrate analogs (Fig. 4)[23] and the ribonuclease inhibitor protein (RI).[32,33]

Assays are performed with stirring in 2.00 ml of 0.10 *M* MES–NaOH buffer (pH 6.0) containing NaCl (0.10 *M*), substrate (60 n*M*), and enzyme (5–500 p*M*) [plus dithiothreitol (5 m*M*) for RI K_i determination]. The inhibitor stock solution should be prepared in the same assay buffer. The value of $\Delta F/\Delta t$ is measured for 5 min after enzyme is added. Next, an aliquot of inhibitor is added, and $\Delta F/\Delta t$ of the newly established linear region determined, now in the presence of inhibitor. The concentration of inhibitor in the assay is doubled repeatedly in 5-min intervals until $\Delta F/\Delta t$ decreases to less than 10% of the initial uninhibited value. If the volume increase caused by adding the inhibitor is significant, the concentration of the enzyme and the substrate should be corrected by considering the dilution. For optimal results, the inhibitor concentrations in the reaction should be planned to span the range from $<K_i/10$ to >10 K_i. (It is convenient to prepare a stock solution of inhibitor with a concentration 100-fold greater than K_i.) To obtain a properly weighted set of data for statistical analysis, the inhibitor concentration in the reaction should be increased exponentially by increasing the volume of each inhibitor addition. To ensure that the entire inhibition assay occurs during steady-state conditions, excess ribonuclease is then added to the reaction mixture so as to cleave all substrate. This addition is done to obtain the final fluorescence intensity and to confirm that less than 10% of the substrate is cleaved prior to completion

[32] L. E. Bretscher, R. L. Abel, and R. T. Raines, *J. Biol. Chem.* **275,** 9893 (2000).
[33] T. A. Klink and R. T. Raines, *J. Biol. Chem.* **275,** 17463 (2000).

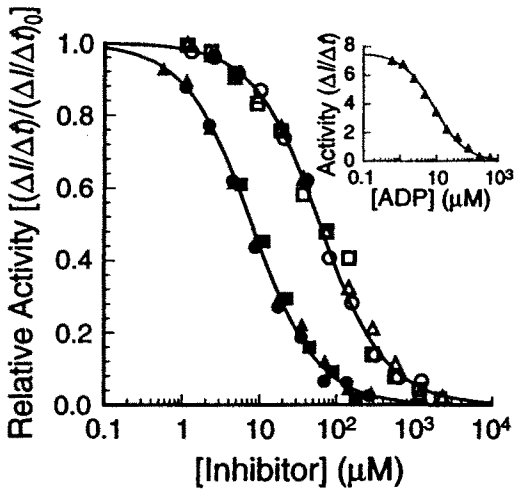


FIG. 4. Competitive inhibition of ribonuclease A catalysis by uridine 3′-phosphate (open symbols) and adenosine 5′-diphosphate (closed symbols). Relative activity is the ratio of the slope determined at each concentration of inhibitor to the slope determined without any inhibitor. Triangle, square, and circle symbols represent three different sets of data determined independently. Absolute activity in one set of data. Data were fitted to Eq. (4).[23]

of the inhibition assay. The value for K_i is determined by non-linear least squares regression analysis of $\Delta F/\Delta t$ fitted to Eq. (4):

$$\Delta F/\Delta t = (\Delta F/\Delta t)_0 \left(\frac{K_i}{[I] + K_i} \right) \tag{4}$$

In Eq. (4), $(\Delta F/\Delta t)_0$ is the initial slope prior to the addition of inhibitor.

Determination of K_i values can be a useful tool for detecting a contaminating ribonuclease. Traditionally, a protein is considered to be pure if it constitutes >95% of the protein visible after polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate (SDS–PAGE). Analysis by SDS–PAGE is insufficient, however, if the contaminating enzyme is a more efficient catalyst than the test enzyme. For example, consider the inadvertent contamination of angiogenin by RNase A. RNase A is $>10^5$-fold better catalyst of RNA cleavage than is angiogenin (Table II). If RNase A is a 0.01% contaminant in an angiogenin preparation, then the determined K_i value will be similar to that for RNase A rather than the actual value of angiogenin because >90% of the ribonucleolytic activity is from the contaminant. The K_i value, along with SDS–PAGE and zymogram assays, can be used to show that angiogenin is effectively free of ribonucleolytic activity from contaminating RNase A.[23]

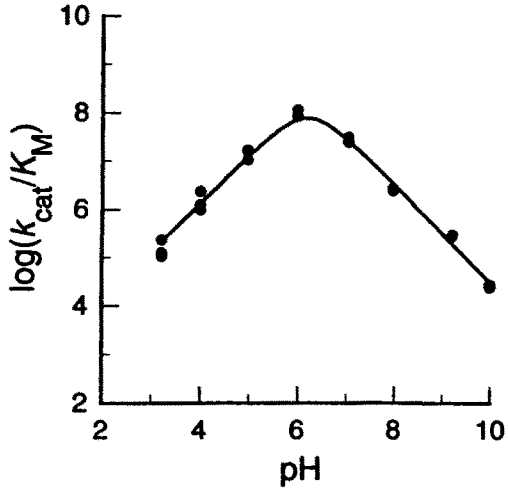


FIG. 5. pH–Rate profile for catalysis of 6-FAM–$(dA)_2rU(dA)_3$–6-TAMRA cleavage by ribonuclease A. Values of k_{cat}/K_m were determined in 0.10 *M* buffer containing NaCl (0.10 *M*) and 6-FAM–$dArU(dA)_2$–DABCYL (5–80 n*M*). Buffers were sodium citrate (pH 3.22), sodium succinate (pH 3.84 and 4.97), MES–NaOH (pH 5.99), MOPS–NaOH (pH 7.04), Tris-HCl (pH 7.97), CHES–NaOH (pH 9.14), and CAPS–NaOH (pH 10.0). Data were fitted to Eq. (5).[37]

pH–Rate Profiles

pH–Rate profiles have been used often and with great success to reveal the role of acidic and basic functional groups in catalysis.[34–36] To obviate artifacts from changing pH, the substrate used in the determination of a pH–rate profile should be stable within the pH range used for the experiments. A 6-TAMRA label is not optimal for pH–rate profiles because of its chemical instability at acidic pH. The pH–rate profile for catalysis by wild-type RNase A, has, however, been determined with 6-FAM–$dArU(dA)_2$–4-DABCYL because the 4-((4-(dimethylamino)phenyl)azo)benzoic acid (DABCYL) label is a stable quencher throughout the pH range (Fig. 5). Also, the fluorescence intensity of fluorescein is sensitive to pH because the anionic form of the molecule has greater fluorescence than does the neutral form ($pK_a \sim 7.5$). As a consequence, a higher concentration of the substrate is required for assaying activity at acidic pH (40–80 n*M*) than at basic pH (5–10 n*M*). Acid dissociation constants were determined to be 6.0 and 6.4

[34] J. R. Knowles, *CRC Crit. Rev. Biochem.* **4**, 165 (1976).
[35] W. W. Cleland, *Methods Enzymol.* **87**, 390 (1982).
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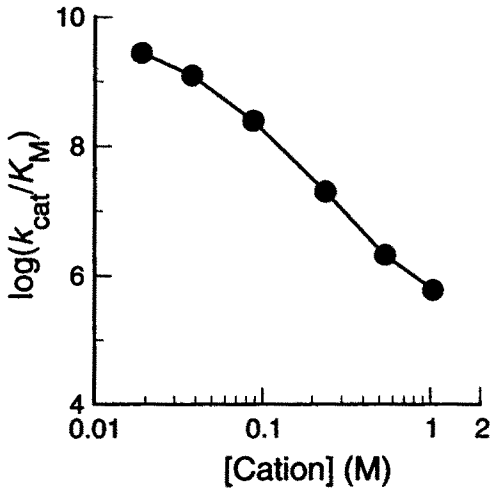

FIG. 6. Salt–rate profile for catalysis of 6-FAM–$(dA)_2rU(dA)_3$–6-TAMRA cleavage by ribonuclease A. Values of k_{cat}/K_m were determined in 25 m*M* or 50 m*M* Bis–Tris-HCl (pH 6.0) containing NaCl (0–1.0 *M*) and 6-FAM–$(dA)_2rU(dA)_3$–6-TAMRA (20 n*M*). [Cation] is the sum of the concentration of Bis–Tris cation and Na^+.[40]

by fitting the pH–rate profile in Fig. 5 to Eq. (5) by nonlinear regression analysis.[37]

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_0}{1 + 10^{(pH-pK_1)} + 10^{(pK_2-pH)}} \quad (5)$$

Salt–Rate Profiles

Though less often used than pH–rate profiles, salt–rate profiles can provide valuable insights into the role of Coulombic interactions in catalysis. Such interactions are known to be of great importance in the interaction of ribonucleases with their substrates.[38–40] The determination of k_{cat}/K_m values for ribonucleases at low salt concentration is problematic because K_m decreases significantly at low salt. Moreover, kinetic analyses could be complicated because of product or substrate inhibition at low salt.[41] The fluorogenic substrates can eliminate this pitfall because k_{cat}/K_m can be determined with a low concentration of substrates.

The salt–rate profile for catalysis by RNase A has been determined with 6-FAM–$(dA)_2rU(dA)_3$–6-TAMRA in 25 m*M* or 50 m*M* Bis–Tris-HCl buffer

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(pH 6.0) containing NaCl (0–1.0 *M*) (Fig. 6).[40] The k_{cat}/K_m of RNase A is $> 10^9$ $M^{-1}s^{-1}$ at low salt and decreases monotonically as salt concentration increases. Special care in preparing buffer solution should be taken for assays at low salt to avoid possible inhibition by low-level contaminants in common buffers. Because enhanced Coulombic interactions at low salt decrease K_i of inhibitory contaminants significantly, more than 10^4-fold inhibition was observed in certain types of buffers at low salt concentration.[40]

S·Tag Fusion System

The N-terminal 15 amino acid residues of RNase A (S·Tag) are an effective carrier in protein fusion systems.[10,42] This pentadecapeptide will bind to the C-terminal 104 amino acid residues of RNase A to produce an enzyme with high ribonucleolytic activity. [Reagents to use the S·Tag Fusion System are available commercially from Novagen (Madison, WI; *http://www.novagen.com*).] The hypersensitive assays described herein enable the detection of extremely low levels of proteins with a fused S·Tag. Moreover, because assays based on fluorescence spectroscopy are easy to automate, the fluorogenic substrates facilitate the high-throughput screening of proteins having an S·Tag.

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