Communications to the Editor

Value of General Acid–Base Catalysis to Ribonuclease A

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Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) has been one of the most studied of all enzymes.[1] RNase A efficiently catalyzes the cleavage of RNA.[2] Early X-ray diffraction analyses revealed that the active site of RNase A contains two histidine residues, histidine 12 (H12) and histidine 119 (H119).[3] The results of chemical modification[4] and pH–rate[5] studies are consistent with an enzymatic reaction mechanism in which the rate-limiting transition state for RNA cleavage is similar to that shown in Figure 1. In this mechanism, the imidazole side chain of H12 acts as a general base by deprotonating the 2′ oxygen, and that of H119 acts as a general acid by protonating the 5″ oxygen. These two residues have evoked much interest in bioorganic chemistry,[6] as well as in protein chemistry and enzymology.[1] Indeed, no residue other than H12 and H119 need be invoked to explain the classic bell shape of the pH–rate profile[5] for catalysis by this enzyme. Here, we report the explicit value of this general acid and this general base to catalysis of RNA cleavage by RNase A.

We used recombinant DNA techniques to produce mutant ribonucleases in which either H12 or H119 was changed to an alanine residue.[7] This change effectively substitutes a proton for the imidazole group of each residue. We then determined the ability of the resulting mutant enzymes, H12A RNase A and H119A RNase A, to catalyze the cleavage of three phosphodiester substrates: polycytidylic acid [poly(C)], uridylyl(3′→5′)-adenosine (UpA), and uridine 3′-(*p*-nitrophenyl phosphate) ($UpOC_4H_6$-*p*-NO_2).[9]

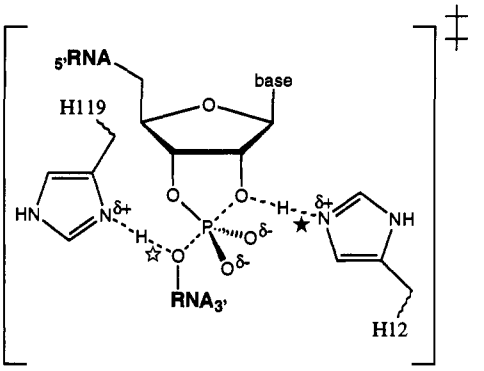

Figure 1. Putative structure of the transition state for the RNase A-catalyzed cleavage of RNA. Proposed interactions with H12 (★) and H119 (☆) are indicated.

The values of the steady-state kinetic parameters for cleavage of poly(C), UpA, and $UpOC_4H_6NO_2$ by the wild-type and mutant ribonucleases are given in Table 1 and Figure 2. The second-order rate constant, k_{cat}/K_m, is proportional to the association constant of an enzyme and the rate-limiting transition state during catalysis.[10] As shown in Figure 2, eliminating the imidazole group of H12 decreased the affinity of the enzyme for this transition state by 10^4-fold during cleavage of poly(C), UpA, and $UpOC_6H_4$-*p*-NO_2.[11] Eliminating the imidazole group of H119 decreased this affinity by 10^4-fold during cleavage of poly(C) and by almost 10^4-fold during cleavage of UpA.[12] In contrast, this change had no significant effect on the rate of cleavage of $UpOC_6H_4NO_2$.

The value of the imidazole group of H119 to catalysis depends on the pK_a of the conjugate acid of the leaving groups. Cleavage of poly(C) and UpA is accelerated dramatically by the side chain of H119. The nucleotide and nucleoside leaving groups in these substrates have conjugate acids with $pK_a \approx 14.8$.[13] In contrast, the cleavage of $UpOC_6H_4$-*p*-NO_2 is unaffected by the side chain of H119. The *p*-nitrophenolate leaving group in the substrate has a conjugate acid with $pK_a = 7.14$.[14] Together, these data provide the first direct evidence that the role of H119 is to protonate the leaving group during RNA cleavage. This result also illustrates how a capable catalyst for cleavage of an activated model substrate (*e.g.*, $UpOC_6H_4$-*p*-NO_2) can lack a component important for cleavage of an unactivated substrate (*e.g.*, RNA).

The results for $UpOC_6H_4$-*p*-NO_2 illuminate the mechanism of catalysis by RNase A. Breslow has proposed that RNase A

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(1) For reviews, see: (a) Richards, F. M.; Wyckoff, H. W. *Enzymes* **1971**, *4*, 647–806. (b) Karpeisky, M. Ya.; Yakovlev, G. I. In *Soviet Scientific Review, Section D, Vol. 2*; Harvard Academic Press: New York, 1981; pp 145–257. (c) Blackburn, P.; Moore, S. *Enzymes* **1982**, *15*, 317–433. (d) Wlodawer, A. In *Biological Macromolecules and Assemblies, Vol. II, Nucleic Acids and Interactive Proteins*; Jurnak, F. A., McPherson, A., Eds.; Wiley: New York, 1985; pp 393–439. (e) Beintema, J. J. *Life Chem. Rep.* **1987**, *4*, 333–389. (f) Eftink, M. R.; Biltonen, R. L. In *Hydrolytic Enzymes*; Neuberger, A., Brocklehurst, K., Eds.; Elsevier: New York, 1987; pp 333–376.

(2) The enzyme also catalyzes (albeit inefficiently, see: Thompson, J. E.; Venegas, F. D.; Raines, R. T. *Biochemistry*, in press) the hydrolysis of the 2′,3′-cyclic phosphodiester product of RNA cleavage.

(3) (a) Kartha, G.; Bello, J.; Harker, D. *Nature* **1967**, *213*, 862–865. (b) Avey, H. P.; Boles, M. O.; Carlisle, C. H.; Evans, S. A.; Morris, S. J.; Palmer, R. A.; Woolhouse, B. A.; Shall, S. *Nature* **1967**, *213*, 557–562.

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(6) (a) Breslow, R.; Labelle, M. *J. Am. Chem. Soc.* **1986**, *108*, 2655–2659. (b) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473–4482. (c) Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317–324. (d) Breslow, R.; Anslyn, E.; Huang, D.-L. *Tetrahedron* **1991**, *47*, 2365–2376. (e) Breslow, R.; Xu, R. *J. Am. Chem. Soc.* **1993**, *115*, 10705–10713.

(7) Mutations in the cDNA that codes for RNase A were made by oligonucleotide-mediated site-directed mutagenesis (Kunkel, T. A.; Roberts, J. D.; Zakour, R. A. *Methods Enzymol.* **1987**, *154*, 367–382). Mutant cDNAs were expressed in *Escherichia coli* under the control of the T7 RNA polymerase promoter, and the resulting proteins were purified by standard methods (ref 8).

(8) delCardayré, S. B.; Raines, R. T. *Biochemistry* **1994**, *33*, 6031–6037.

(9) Poly(C) was from Sigma Chemical (St. Louis, MO) and was purified by precipitation from aqueous ethanol (70% v/v). UpA was synthesized by the methods of Ogilvie (Ogilvie, K. K.; Beaucage, S. L.; Schifman, A. L.; Theriault, N. Y.; Sadana, K. L. *Can. J. Chem.* **1978**, *56*, 2768–2780) and Carruthers (Beaucage, S. L.; Carruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859–1862). $UpOC_4H_6$-*p*-NO_2 was synthesized by the method of Williams (Davis, A. M.; Regan, A. C.; Williams, A. *Biochemistry* **1988**, *27*, 9042–9047).

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(11) The kinetic parameters in Table 1 for $UpOC_6H_4$-*p*-NO_2 cleavage are about 2-fold higher than those reported previously (Davis, A. M.; Regan, A. C.; Williams, A. *Biochemistry* **1988**, *27*, 9042–9047). This disparity may result from a difference in the pH of the assay solutions (6.0 vs 7.5).

(12) Replacing H119 of bovine seminal ribonuclease (a homolog of RNase A) with an aspartate residue decreases k_{cat}/K_m by (4×10^3)-fold for UpA cleavage (Kim, J.-S.; Raines, R. T., unpublished results). The H13A and H114A mutants of human angiogenin (another homolog of RNase A) each have $>10^4$-fold less activity for RNA cleavage than does wild-type human angiogenin (Shapiro, R.; Vallee, B. L. *Biochemistry* **1989**, *28*, 7401–7408).

(13) $CH_3OCH_2CH_2OH$ has $pK_a = 14.8$ (Ballinger, P.; Long, F. A. *J. Am. Chem. Soc.* **1960**, *82*, 795–798).

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Table 1. Steady-State Kinetic Parameters for Cleavage of Ribonucleotides by Wild-Type and Mutant Ribonucleases[a]

RNase A	substrate[b]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_{cat}/K_m)^{mutant}/(k_{cat}/K_m)^{wild\text{-}type}$
wild-type	poly(C)	$(4.1 \pm 0.1) \times 10^2$ [c]	0.034 ± 0.002[c]	$(1.5 \pm 0.1) \times 10^7$ [c]	1.0
H12A	poly(C)	0.073 ± 0.006	0.105 ± 0.025	$(7.3 \pm 1.2) \times 10^2$	$(4.9 \pm 0.9) \times 10^{-5}$
H119A	poly(C)	0.24 ± 0.03	0.21 ± 0.03	$(1.1 \pm 0.1) \times 10^3$	$(7.3 \pm 0.8) \times 10^{-5}$
wild-type	UpA	$(1.40 \pm 0.15) \times 10^3$ [c]	0.62 ± 0.09[c]	$(2.3 \pm 0.4) \times 10^6$ [c]	1.0
H12A	UpA	0.15 ± 0.02	0.86 ± 0.17	$(1.7 \pm 0.1) \times 10^2$	$(7.4 \pm 1.4) \times 10^{-5}$
H119A	UpA	0.76 ± 0.10	0.80 ± 0.15	$(9.5 \pm 0.5) \times 10^2$	$(4.1 \pm 0.7) \times 10^{-4}$
wild-type	$UpOC_6H_4$-*p*-NO_2	18.8 ± 0.6	0.33 ± 0.05	$(5.7 \pm 0.6) \times 10^4$	1.0
H12A	$UpOC_6H_4$-*p*-NO_2	0.0029 ± 0.0001	0.275 ± 0.041	$(1.1 \pm 0.1) \times 10^1$	$(1.9 \pm 0.3) \times 10^{-4}$
H119A	$UpOC_6H_4$-*p*-NO_2	27 ± 1	0.76 ± 0.08	$(3.6 \pm 0.1) \times 10^4$	0.63 ± 0.11

[a] All reactions were performed at 25 °C in 50 mM MES buffer, pH 6.0, containing 0.1 M NaCl. Steady-state kinetic parameters were determined by fitting initial velocity data to a hyperbolic curve using the program HYPERO.[22] [b] Cleavage of poly(C) and $UpOC_6H_4$-*p*-NO_2 were monitored at 250 nm ($\Delta\epsilon_{250}$ = 2380 M^{-1} cm^{-1}) and 330 nm ($\Delta\epsilon_{330}$ = 4560 M^{-1} cm^{-1}), respectively; cleavage of UpA was monitored at 265 nm in the presence of excess adenosine deaminase[23] ($\Delta\epsilon_{265}$ = −6000 M^{-1} cm^{-1}). [c] Data from ref 8.

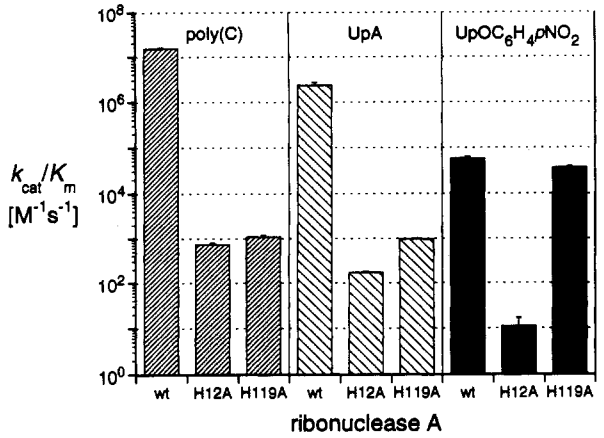

Figure 2. Values of k_{cat}/K_m for the cleavage reaction catalyzed by wild-type and mutant ribonucleases. [8]

catalyzes RNA cleavage via a phosphorane intermediate.[6b] In the Breslow mechanism, H119 is proposed to both protonate a nonbridging oxygen of the phosphate anion and deprotonate this same oxygen in the phosphorane intermediate.[15] Yet, wild-type and H119A RNase A cleaved $UpOC_6H_4$-*p*-NO_2 at the same rate (Figure 2). Thus our data argue against the participation of H119 in the formation of a phosphorane, at least during the cleavage of $UpOC_6H_4$-*p*-NO_2.[16]

The steady-state kinetic parameters of wild-type, H12A, and H119A RNase A are consistent with H12 acting as a general base and show that H119 acts as a general acid during the cleavage of RNA. Further, the observed rate enhancements agree with those expected for acid–base catalysis by H12 and H119. For example, suppose a water molecule replaces the imidazole group in the mutant enzymes such that the interactions marked by ★ or ☆ in Figure 1 are now to the oxygen or a hydrogen, respectively, of H_2O. The rate enhancements then derived from the Brønsted equation are $k_{wt}/k_{H12A} = (K_a^{H_3O^+}/K_a^{H12})^{\beta}$ and $k_{wt}/k_{H119A} = K_a^{H119}/K_a^{H_2O})^{\alpha}$, where $pK_a^{H12} = 5.8$ and $pK_a^{H119} = 6.2$,[17] and $pK_a^{H_3O+} = -1.7$ and $pK_a^{H_2O} = 15.7$. The Brønsted equation therefore predicts that general base catalysis provides a $10^{7.5\beta}$-fold rate enhancement, and general acid catalysis provides a $10^{9.5\alpha}$-fold rate enhancement. Values of α and β tend to be approximately 0.5 for proton transfers between oxygen and nitrogen.[18] Thus, the rate enhancements predicted with this simple model are similar to those observed (Figure 2).

The side chains of H12 and H119 each bind to the rate-limiting transition state during RNA cleavage with an apparent free energy of ΔG_{app} = 5–6 kcal/mol.[19] This free energy is related[20] to the strength of the interaction marked by ★ or ☆ in Figure 1. For many reasons, however, neither of these interactions can be assigned an explicit free energy. For example, mutagenesis may have altered the structure of the transition state or its position on the reaction coordinate.[10,18,20] Also, catalysis by wild-type RNase A may be limited in part by a diffusive step rather than a chemical interconversion.[21] Studies to illuminate these and other aspects of catalysis by this venerable enzyme are underway.

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(15) In the Breslow mechanism, H119 is also proposed to protonate the leaving group during breakdown of the phosphorane intermediate. This role is probably unnecessary, however, if *p*-nitrophenolate is the leaving group.

(16) Gerlt and Gassman have proposed the existence of the same phosphorane intermediate as in the Breslow mechanism (Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943–11952). In the Gerlt and Gassman mechanism, however, H119 serves only to protonate the 5″ oxygen, a role that is not in conflict with our results.

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(20) Fersht, A. R. *Biochemistry* **1988**, *27*, 1577–1580.
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