

Ribonuclease A: from Model System to Cancer Chemotherapeutic

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ABSTRACT: Ribonuclease A has been the most studied enzyme of the twentieth century. Work on ribonuclease A has endowed enzymology, as well as protein science, with many paradigms. This work continues to provide a framework for understanding protein structure – function relationships in atomic detail. In addition, knowledge gained by basic research is enabling the rapid deployment of ribonuclease A homologs and variants as chemotherapeutic agents for the treatment of cancer and other human diseases.

For the last fifty years, chemists and biochemists have been investigating the origins of the extraordinary effectiveness of enzymes as catalysts. The aims have been to identify the reaction intermediates, to measure the rates of their interconversion, and to determine the precise role of amino acid residues in the process. A variety of enzymes have contributed to this understanding. One of these enzymes is bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5).

RNase A has been the object of landmark work in enzymology, as well as on the folding, stability, and chemistry of proteins, and on molecular evolution [1,2]. The seminal contributions of RNase A to chemistry was recognized by the awarding of Nobel Prizes to Anfinsen, Stein, and Moore in 1972, and to Merrifield in 1984. Each of these laureates chose RNase A as his model system.

1. Structure

RNase A was first crystallized over fifty years ago, and these crystals were shown to diffract to a resolution of 2 Å. RNase A was the first enzyme and third protein (after insulin and hemoglobin) for which a correct amino acid sequence was determined, and the third enzyme and fourth protein (after myoglobin, lysozyme, and carboxypeptidase A) whose three-dimensional structure was determined by X-ray diffraction analysis. More recently, work on RNase A has yielded the first three-dimensional structure of a protein containing an isoaspartyl residue, which derives from the deamidation of an asparagine residue (here, Asn67). Finally,

the use of NMR spectroscopy in elaborating both protein structure and protein folding pathway were developed with RNase A. The ^1H -NMR resonances of the enzyme have been assigned, and the structure of the enzyme in aqueous solution has been determined. Altogether, over 70 sets of three-dimensional coordinates related to RNase A have been deposited in the Brookhaven Protein Data Bank (www.pdb.bnl.gov), and this number is growing with the deposition of coordinates of RNase A variants [3].

RNase A is small. The mature enzyme, as secreted by exocrine cells of the bovine pancreas, has only 124 amino acid residues. RNase A contains 19 of the 20 natural amino acids, lacking only tryptophan. The molecular formula of the native, uncharged enzyme is $\text{C}_{575}\text{H}_{907}\text{N}_{171}\text{O}_{192}\text{S}_{12}$, corresponding to a molecular mass of 13,686 Da.

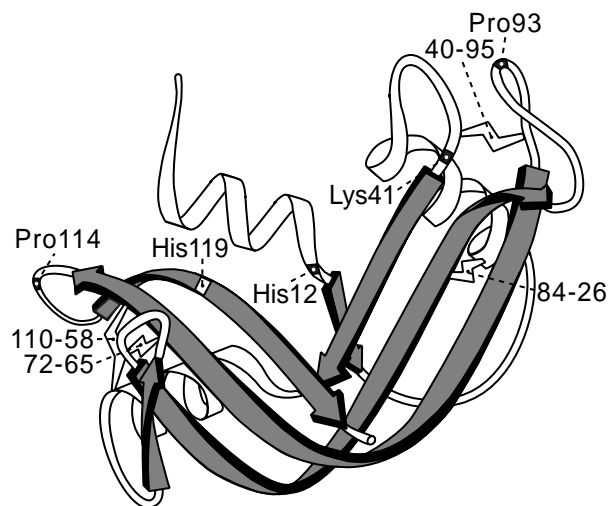
The overall shape of the enzyme resembles that of a kidney, with the active-site residues lying in the cleft (Figure 1). The predominant elements of secondary structure are a long four-stranded antiparallel β -sheet and three short α -helices. The enzyme is crosslinked by four disulfide bonds, which involve all eight of its cysteine residues. The peptide bonds preceding two of the four proline residues are in the *cis* conformation. These proline residues are in type VI reverse turns at opposite ends of the native enzyme.

2. Mechanism of Catalysis

RNase A catalyzes the hydrolysis of $\text{P}-\text{O}^{5'}$ bonds in RNA. Figure 2 depicts a mechanism of catalysis that is consistent with all known data from work on the enzyme itself. In the mechanism in Figure 2, the side chain of His12 acts as a base that abstracts a proton from the 2'-oxygen of a substrate molecule, and thereby facilitates its attack on the phosphorus atom. This attack proceeds in-line to displace a nucleoside. The side chain of His119 acts as an acid that protonates the 5''-oxygen to facilitate its displacement. Both products are released to solvent. The slow hydrolysis of the 2',3'-cyclic phosphodiester occurs in a separate process [4], and resembles the reverse of transphosphorylation.

The reactions in Figure 2 probably occur via transition states having a pentavalent phosphorus atom, as shown in Figure 3. The side chain of Lys41 and the main chain of Phe120

Figure 1. Ribbon diagram of the three-dimensional structure of ribonuclease A. The inscriptions refer to the location of the eight cysteine residues that give rise to the four disulfide bonds, two proline residues with *cis* peptide bonds, and the three residues most important for catalysis: His12, His119, and Lys41.



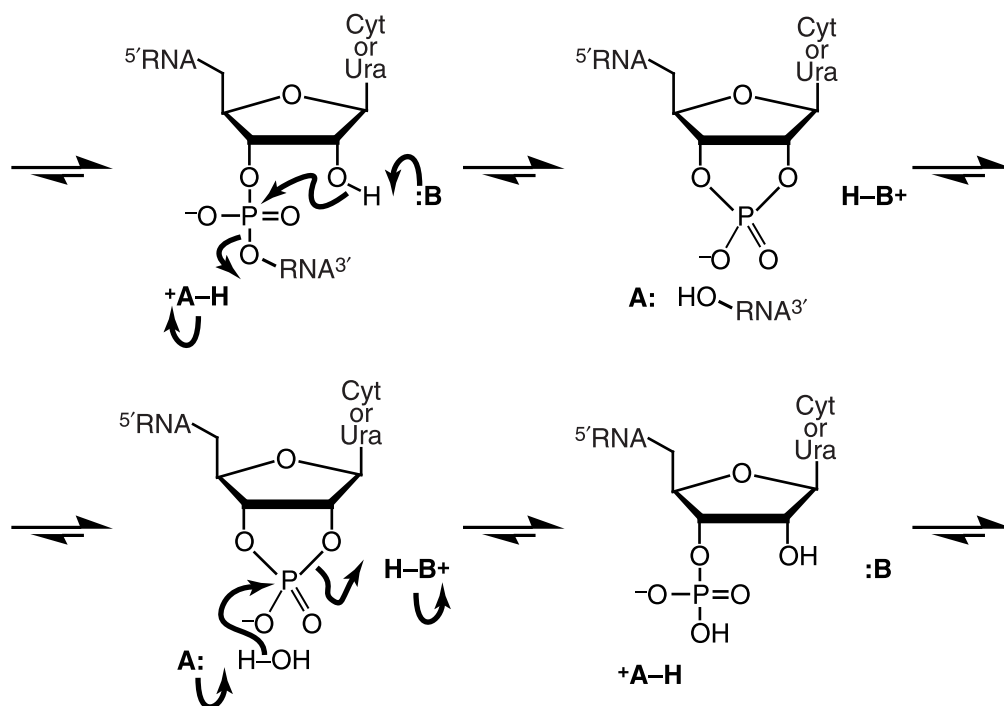


Figure 2. Putative mechanism of the transphosphorylation reaction (top) and hydrolysis reaction (bottom) catalyzed by ribonuclease A. “B” is His12, and “A” is His 119.

enhance catalysis by stabilizing this transition state. The high-resolution structure of the crystalline complex of RNase A and U>v obtained by joint X-ray/neutron diffraction analysis has provided valuable insight into the catalytic mechanism of RNase A [5]. The active site of this structure is shown in Figure 4. In the active site, the side chains of His12, His119, Lys41, and Gln11, and the main chain of Phe120 are all proximal to the vanadyl group. The main-chain nitrogen of Phe120 donates a hydrogen bond to a nonbridging oxygen. The roles of Gln11 [6], His12 [7], Lys41 [8,9], His119 [7], and Asp121 [10] have been probed in detail by site-directed mutagenesis followed by extensive kinetic analyses. No data exist on the role of the main-chain nitrogen of Phe120 in catalysis.

The results of experiments in imidazole buffer (but in the absence of enzyme) have been used to argue for a triester mechanism

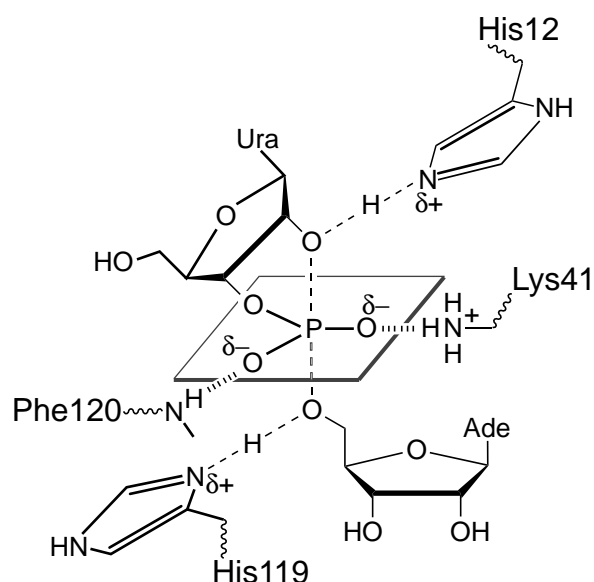


Figure 3. Putative structure of the transition state during transphosphorylation of UpA by ribonuclease A. The dissociation constant for this complex is $K_{TX} \leq 10^{-14.7}$ M [9].

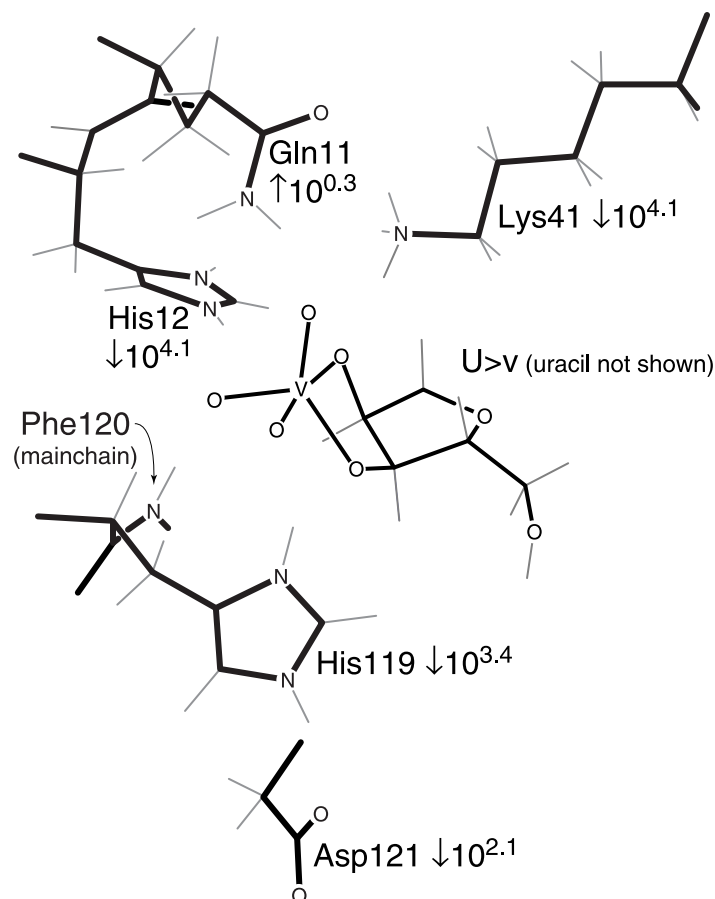


Figure 4. Crystalline structure of the active site of ribonuclease A bound to uridine 2',3'-cyclic vanadate (U>v), a transition state analog [5]. The structure was refined at 2.0 Å from X-ray and neutron diffraction data. The number near each side chain refers to the effect of replacing that residue with alanine on the value of $k_{\text{cat}}/K_{\text{m}}$ for UpA cleavage [6–10].

of catalysis [11]. In this mechanism, His119 is proposed to both protonate a nonbridging oxygen of the phosphoryl group and deprotonate this same oxygen in a phosphorane intermediate. The results of at least two recent experiments on the enzyme itself provide direct evidence against this view. First, wild-type RNase A and the H119A variant cleave $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$ at the same rate [7]. These data preclude the participation of His119 in the formation or breakdown of a phosphorane, at least during the cleavage of $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$. Secondly, kinetic isotope effect data on the cleavage of ^{18}O -labelled $\text{UpOCH}_2\text{C}_6\text{H}_4\text{-}m\text{-NO}_2$ by RNase A are inconsistent with a triester mechanism [12]. Rather, these data support a concerted mechanism in which the transition state is slightly associative.

Why does RNase A not use the triester mechanism? In the active site of RNase A, the desolvated side chains of His12 and His119 are aligned to interact simultaneously as a base and acid with a bound, desolvated substrate (Figure 3). Such an alignment of two imidazolyl groups is implausible in imidazole buffer and improbable in an enzyme mimic. Thus, the enzyme can access a reaction coordinate that is simply unavailable in nonenzymic systems.

3. Energetics of Catalysis

The energetics of catalysis by RNase A are not yet characterized completely. Like proteases, ribonucleases catalyze exergonic reactions. Monitoring the reverse of the transphosphorylation and hydrolysis reactions is difficult. The revelation of the reaction energetics of ribonuclease catalysis is therefore more challenging than is that of an enzyme that catalyzes a relatively isogonic interconversion. Nonetheless, progress has been made.

The first consideration in analyzing the reaction energetics of RNase A is throughput. In other words, how much of the 2',3'-cyclic phosphodiester product from the first step undergoes hydrolysis in the second step without dissociating from the enzyme? Experiments in which the turnover of [5,6-³H]UpA was monitored in the presence of excess unlabeled U>p indicate that only 0.1% of an RNA substrate is *both* transphosphorylated *and* hydrolyzed without dissociating [4]. Thus, the mechanism of RNA hydrolysis by RNase A proceeds in two one-step processes, rather than in one two-step process.

The existence of two discrete steps has an important implication for the mechanism of the reaction catalyzed by RNase A. The imidazole group of His12 acts as a base in the transphosphorylation reaction and as an acid in the hydrolysis reaction. The imidazole group of His119 has a complementary role, acting as an acid in the transphosphorylation reaction and a base in the hydrolysis reaction. After catalysis of transphosphorylation, each histidine residue in the active site of RNase A is protonated appropriately to catalyze hydrolysis of the bound cyclic intermediate. After hydrolysis of this substrate, each histidine residue is returned to its initial protonation state, completing the catalytic cycle. Yet, RNase A short-circuits this cycle by releasing, rather than hydrolyzing, the cyclic intermediate. Thus, RNase A has an iso mechanism, in which protonation states of the unliganded enzyme are interconverted by a pathway that does not involve substrate molecules.

The products of the uncatalyzed cleavage of UpA are the same as those in the RNase A-catalyzed reaction. The identity of these reaction products is consistent with the uncatalyzed and catalyzed transphosphorylation reactions proceeding by the same mechanism. If a reaction does proceed by the same mechanism in the absence and presence of an enzyme, then the ratio of $k_{\text{cat}}/K_{\text{m}}$ for the enzyme-catalyzed reaction to k_{uncat} for the uncatalyzed reaction provides a measure of the affinity of the enzyme for the rate-limiting transition state during catalysis. At pH 6.0 and 25 °C, RNase A catalyzes the transphosphorylation of UpA with a $k_{\text{cat}}/K_{\text{m}}$ of $2.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ [6]. Under identical conditions, the uncatalyzed rate of UpA transphosphorylation, measured by following the cleavage of [5,6-³H]Up[3,5,8-³H]A for several weeks, is $5 \times 10^{-9} \text{ s}^{-1}$ [12]. This rate constant corresponds to a $t_{1/2}$ of 4 y for the P–O^{5'} bond in UpA. (The $t_{1/2}$ of the P–O^{5'} bonds in RNA stems and loops is likely to be much larger because of improper alignment for in-line attack by the 2'-oxygen.) The dissociation constant for the rate-limiting transition state during the transphosphorylation of UpA is therefore $K_{\text{TX}} = 10^{-14.7} \text{ M}$. Because the rate-limiting transition state may not involve a change in covalency, this value for K_{TX} is an upper limit for the dissociation constant of the enzyme bound to the chemical transition state for P–O^{5'} bond cleavage (Figure 3). Data on the catalyzed and uncatalyzed and RNase A-catalyzed hydrolysis of UpA can be compiled into a free energy profile, as shown in Figure 5. These data, along with the low throughput of the enzymatic reaction, suggest that RNase A has evolved primarily to catalyze transphosphorylation rather than hydrolysis. The manifestation of this preference is likely to reside in the enzymic

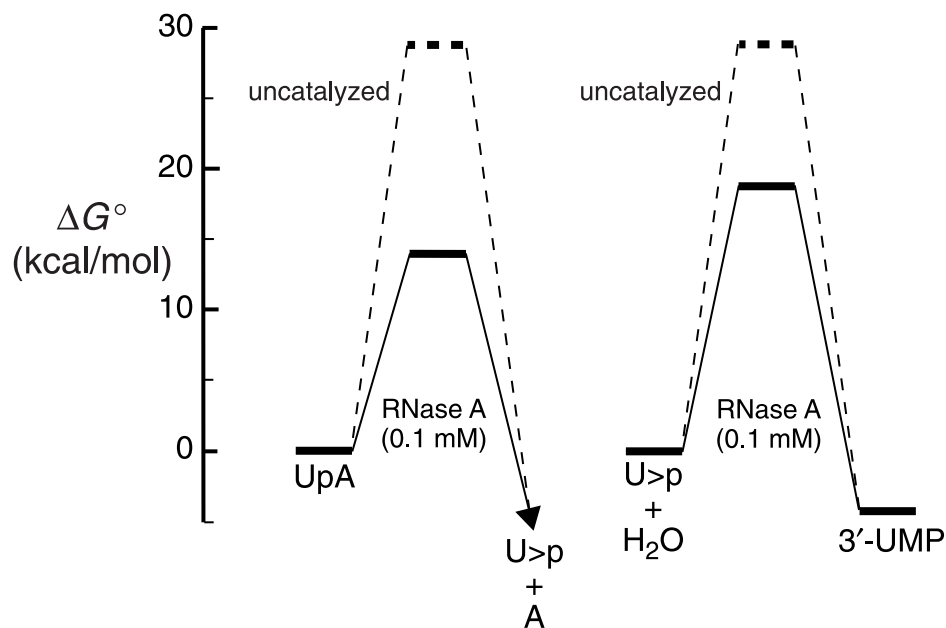


Figure 5. Free energies for the uncatalyzed (– –) and ribonuclease A-catalyzed (—) transphosphorylation of UpA (left) and hydrolysis of U>p (right) [9]. Free energies of activation were calculated for the reaction at pH 6.0 and 25 °C with the equation: $\Delta G^\ddagger = -RT \ln[kh/(k_b T)]$ and the values of k_{cat}/K_m and k_{cat} for UpA transphosphorylation, k_{cat}/K_m for U>p hydrolysis, and k_{uncat} for C>p hydrolysis. The free energy of uridine 3'-phosphate (3'-UMP) relative to that of U>p was calculated for the reaction at pH 6.0 and 25 °C with the equation: $\Delta G^\circ = -RT \ln K$, where $K = 1.0 \times 10^3$. The free energies for the ribonuclease A-catalyzed reactions are drawn for a standard state of 0.1 mM, which is the concentration of ribonuclease A in the bovine pancreas. The uncatalyzed hydrolysis of U>p also produces uridine 2'-phosphate in a reaction that is not shown.

subsites (vide infra), which bind nucleotides, but not water, effectively. To denote the more efficacious catalysis of transphosphorylation, perhaps RNase A should be referred to as an “RNA depolymerase”!

4. Substrate Specificity

Structural and functional data divulge the existence of several subsites in RNase A (Figure 6). Three of the enzymic subsites (B1, B2, and B3) interact with the bases of a bound substrate. The B1 subsite appears to bind only pyrimidine bases, and demonstrates a 30-fold kinetic preference for cytidine versus uridine substrates. In contrast, the B2 and B3 subsites bind all bases, but B2 has a preference for an adenine base and B3 has a preference for a purine base. (The B3 “subsite” could result from $\pi - \pi$ stacking interactions that stabilize the enzyme – nucleic acid complex by preorganization or desolvation of the nucleic acid.) Four other enzymic subsites [P(-1), P(0), P(1), and P2] interact with the phosphoryl groups of a bound substrate [13,14]. The enzyme catalyzes the cleavage of the P–O^{5'} bond of a phos-

phoryl group bound in the P1 subsite, which is the active site (Figures 3 and 4).

The side-chain hydroxyl and main-chain carbonyl groups of Thr45 mediate the pyrimidine specificity of RNase A by forming hydrogen bonds to a pyrimidine base and by excluding sterically a purine base. Replacing Thr45 with a smaller glycine or alanine residue enables RNase A to cleave poly(A) efficiently [15,16]. The T45G and T45A enzymes have 10^5 - and 10^3 -fold increases, respectively, in poly(A):poly(C) specificity with little compromise to catalytic efficacy.

The cleavage of poly(A) by both T45G RNase A and T45A RNase A is processive [15]. The distributive mechanism of wild-type RNase A is likely to arise from the opposing specificities of the B1 subsite (which does not bind adenine) and the B2 and B3 subsites (which bind cytosine and uracil only weakly). Inducing RNase A to degrade poly(A) processively requires simply changing the specificity of the B1 subsite to match that of the B2 and B3 subsites. This change results in variants that bind (at the B1 position) and cleave a polymer that can remain bound (at the B2 and B3 positions) after catalysis has occurred (Figure 7). Making RNase A into a processive enzyme effected a new paradigm: a processive enzyme has subsites, each specific for a repeating motif within a polymeric substrate.

Though unable to cleave poly(A) processively, wild-type RNase A can diffuse in one dimension along poly(dA) [17]. This ability is lost if the salt concentration is high, as expected from a Coulombic interaction between the cationic enzyme and an anionic nucleic acid. Such facilitated diffusion may enable cytotoxic homologs of RNase A (vide infra) to use the poly(A) tail of mammalian mRNA's as a runway, leading the enzymes to the pyrimidine nucleotides in the indispensable coding region.

5. Cytotoxicity

The biological functions typically ascribed to ribonucleases are to process and turnover cellular RNA, and to degrade dietary RNA. Yet, some homologs of RNase A appear to have

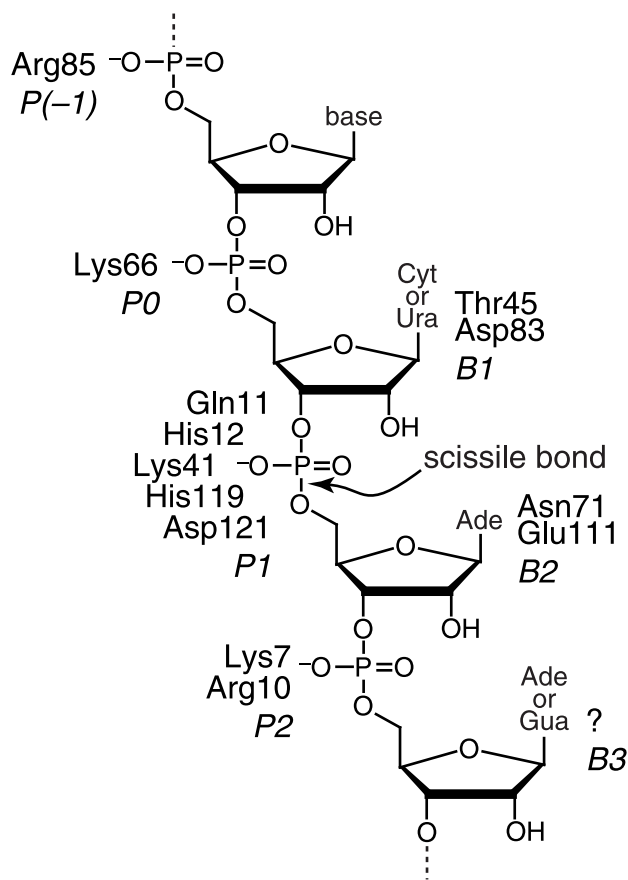


Figure 6. Apparent interactions between the subsites of ribonuclease A and a bound molecule of RNA. The indicated residues have been shown by site-directed mutagenesis to make a contribution to substrate binding or turnover (or both).

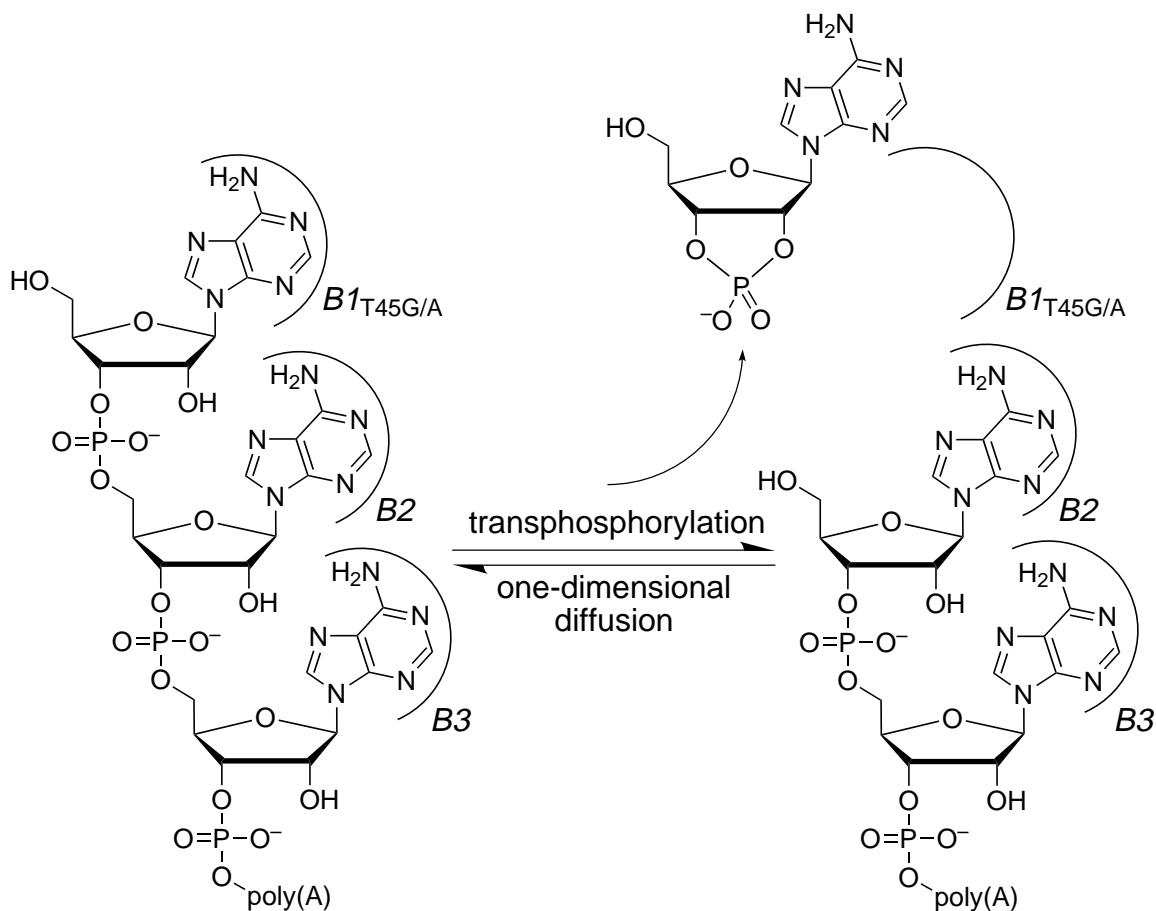


Figure 7. Putative mechanism for the processive cleavage of poly(A) by T45G ribonuclease A and T45A ribonuclease A [15]. The enlarged B1 subsite in these variants can accommodate an adenine base.

quite different roles. These homologs were discovered on the basis of their unusual biological activities. Only later, sometimes much later, were the proteins identified as ribonucleases.

Ribonucleases can be cytotoxic because cleaving RNA renders indecipherable its encoded information. The cytotoxicity of ribonucleases was discovered in the 1950's. Then, RNase A was shown to be toxic to tumor cells, both *in vitro* and *in vivo*. Large doses of RNase A were used in these early studies—effects were observed only after milligrams of enzyme were injected into solid tumors. Smaller doses were found to have no effect.

Over twenty years ago, a homolog of RNase A was discovered in bull seminal plasma that is cytotoxic at low levels. In the last decade, even more cytotoxic homologs were isolated from the eggs of the bullfrog *Rana catesbeiana*, the Japanese rice paddy frog *Rana japonica*, and the Northern leopard frog *Rana pipiens*. All of these *Rana* ribonucleases are toxic to tumor cells *in vitro* with IC₅₀ values near 1 μM. The *Rana pipiens* ribonuclease, called “onconase”, is presently undergoing Phase III human clinical trials for the treatment of pancreatic adenocarcinoma and malignant mesothelioma. If these trials continue to be successful, onconase will be approved for use as a cancer chemotherapeutic in mid-1999. In

addition, onconase has been shown to inhibit HIV-1 replication in chronically infected human cells without killing the virally infected cell [18].

6. Ribonuclease Inhibitor

Ribonuclease inhibitor (RI) is a 50-kDa protein present in the cytosol of mammalian cells. RI contains 15 leucine-rich, $\beta - \alpha$ repeat units arranged symmetrically in a horseshoe. The β -strands form a solvent-exposed β -sheet that defines the inner circumference of RI. The α -helices define the outer surface of the inhibitor. RI forms a 1:1, noncovalent complex with target ribonucleases, including RNase A. Values of the inhibition constant (K_i) are in the fM range. Atomic details of the RI – RNase A interaction are apparent from the three-dimensional structures of the crystalline RI•RNase A complex [19]. In this structure, one-third of RNase A rests within the RI horseshoe and the remainder extends out of the plane defined by the inhibitor.

The inhibitory activity of RI is manifested in the cytosol. This location provides the reducing environment that is necessary to maintain RI activity. Mammalian RI's contain 30 or 32 reduced cysteine residues. Oxidation of a single cysteine residue causes rapid oxidation of the remaining cysteine residues and consequent inactivation of RI. All known RI ligands, including RNase A, are secreted ribonucleases. This observation and the cytosolic localization of RI supports the hypothesis that the inhibitor functions to preserve the integrity of cellular RNA should a secretory ribonuclease inadvertently reach the cytosol.

The protection offered to cells by RI is limited. The cytotoxic activity of bovine seminal ribonuclease (BS-RNase) has been shown to be a consequence of its ability to escape inactivation by RI [20,21]. BS-RNase is isolated as a homodimer, covalently linked by two disul-

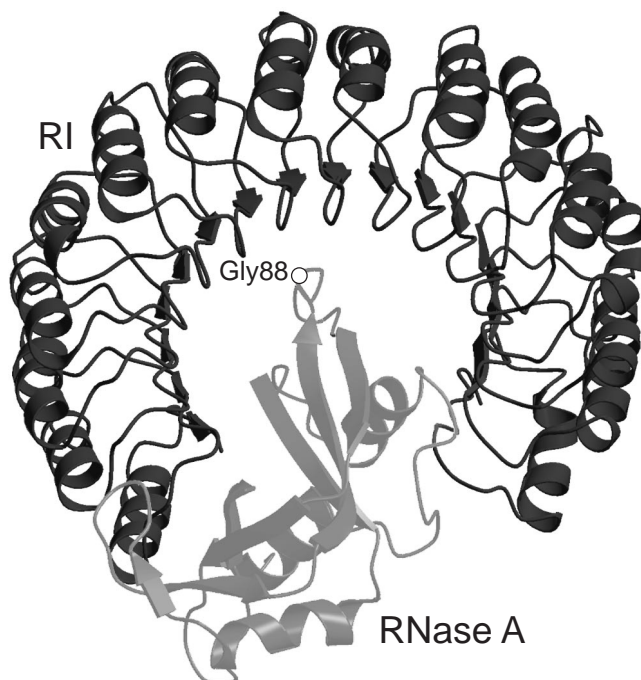


Figure 8. Crystalline structure of the ribonuclease inhibitor•ribonuclease A complex [19], which has a K_i of 67 fM. Gly88 of ribonuclease A, which abuts three tryptophan residues of ribonuclease inhibitor in the complex, was replaced by arginine and aspartate residues in ribonuclease A variants.

vide bonds. As a dimer, BS-RNase is not inhibited by RI and is cytotoxic. RI becomes a potent inhibitor when BS-RNase is reduced to its monomeric form. Though monomeric, onconase also evades tight binding by RI [22]. Onconase retains the elements of tertiary structure that characterize pancreatic-type ribonucleases, but its surface loops are truncated severely compared to their counterparts in RNase A. Further, the majority of RNase A residues that contact RI are replaced by dissimilar residues in onconase.

The relationship between RI inhibition of ribonucleases and ribonuclease cytotoxicity has been investigated directly [23]. Two RNase A variants were created with the intent of introducing steric and electrostatic strain into the RI•RNase A complex without compromising thermal stability or catalytic activity. These goals were achieved by replacing Gly88 with an arginine or aspartate residue (Figure 8; Table 1). Moreover, the G88R and G88D enzymes are toxic to tumor cells (Figure 9; Table 2). Thus, ribonuclease cytotoxicity is a direct consequence of an enzyme's ability to overcome inhibition by RI. Apparently, onconase is not special, as variants of RNase A can manifest similar cytotoxicity. Because RNase A is a mammalian protein and onconase is an amphibian protein, a chemotherapeutic based on RNase A is likely to be less immunogenic and thus more efficacious than is onconase. Finally, appropriate substitutions to the surface loop containing residue 88 in human pancreatic ribonuclease could endow that enzyme with potent cytotoxic activity.

The cytotoxicity of a ribonuclease relies on the delivery of uninhibited ribonucleolytic activity to the cytosol. RI constitutes ~0.01% of the cytosolic protein in a typical mammalian cell. Using this value and data on the volume and composition of the cytosol, the cytosolic concentration of RI can be estimated to be 10^{-6} M. Hence, any ribonuclease with $K_i > 10^{-6}$ M would be largely free (not bound by RI) in the cytosol. The K_i value of onconase is in that range, but those of G88R RNase A and G88D RNase A are not (Table 1). The RNase A variants compensate for being more susceptible to RI by being better catalysts of RNA degradation.

The biological effect of RI can be quantitated by calculating and comparing the ribonucleolytic activity that remains free in the cytosol. Cytotoxicity does not correlate with ribonucleolytic activity. For example, G88D RNase A is a slightly better catalyst than is

Table 1: Biochemical Parameters of Ribonucleases^a

ribonuclease	T_m (°C)	k_{cat} (s ⁻¹)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)	K_i (pM)
wild-type RNase A	63	507	5.7	0.067
G88D RNase A	64	790	3.2	52
G88R RNase A	60	370	2.9	410
onconase	90	0.20	0.014	>10 ⁶

^a T_m is the midpoint of the thermal transition curve. Values of k_{cat} and k_{cat}/K_m are for poly(C) cleavage at pH 6.0 and 25 °C. Values of K_i are for inhibition by ribonuclease inhibitor.

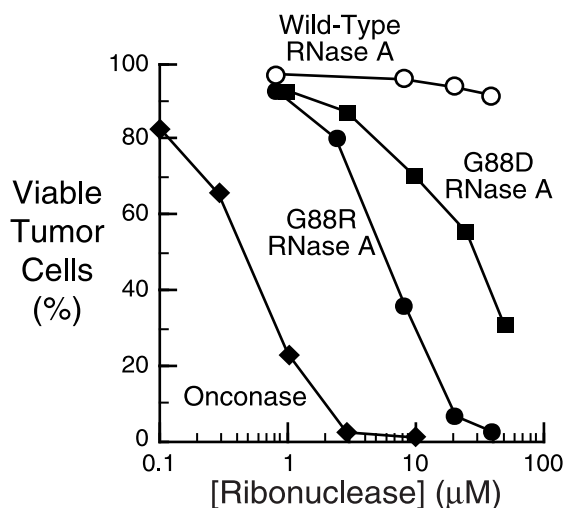


Figure 9. Effect of ribonucleases on the proliferation in culture of K-562 cells, a human leukemia cell line [23]. Proliferation was measured by incorporation of [methyl- ^3H]thymidine into cellular DNA following a 44-h incubation with the ribonucleases. Values reported are the mean from three cultures and are expressed as a percentage of the control, which is the mean from cultures lacking exogenous ribonuclease.

G88R RNase A, but is markedly less cytotoxic. This observation, along with the competitive nature of RI inhibition, indicates that single-stranded RNA is at subsaturating concentrations in the cytosol. In other words, an invading ribonuclease operates under V/K conditions. The second-order rate constant for P–O $^{5'}$ bond cleavage that is relevant *in vivo* can be defined as the *toxicity number*, in analogy to the enzyme’s turnover number:

$$\text{toxicity number} = (k_{\text{cat}}/K_{\text{m}}) / (1 + [\text{RI}]/K_{\text{i}}) \quad (1)$$

The RNA in the cytosol is, of course, heterogeneous. Because all homologs of RNase A catalyze the cleavage of poly(C), the values of $k_{\text{cat}}/K_{\text{m}}$ listed in Table 1 can be used to provide an estimate of the toxicity number. The values thus calculated by using eq 1 are listed in Table 2. These values correlate well with the IC_{50} values—a higher toxicity number corresponds to a lower IC_{50} value (that is, greater cytotoxicity). This correlation suggests that *uninhibited* ribonucleolytic activity is the limiting factor in ribonuclease cytotoxicity.

Table 2: Biological Parameters of Ribonucleases^a

ribonuclease	IC_{50} (μM)	toxicity number ($\text{nM}^{-1}\text{s}^{-1}$)
wild-type RNase A	—	0.0004
G88D RNase A	30	0.2
G88R RNase A	7	0.8
onconase	0.5	>7

^a Values of toxicity number were calculated by using eq 1 and the data in Table 1. Values of IC_{50} were derived from the data shown in Figure 9 [23].

7. Mechanism of Cytotoxicity

The cytotoxicity of a ribonuclease relies on its ribonucleolytic activity [24]. Thus to act as a cytotoxin, a ribonuclease must gain access to cellular RNA. Once in proximity to the RNA, the ribonuclease catalyzes RNA cleavage, resulting ultimately in cell death.

What is the mechanism of cytotoxicity? The process could start by the binding of the enzyme to a cell-surface glycolipid. Several of the *Rana* ribonucleases were first identified not as ribonucleases, but as sialic acid lectins. Sialic acids are displayed on the surface of cells in various forms, notably in gangliosides. Gangliosides are cell-surface glycolipids that contain 1, 2, or 3 sialic acid residues (Figure 10). Gangliosides with high sialic acid content could effectively serve as receptors for cytotoxic ribonucleases. The interaction of ribonucleases with sialic acid residues could occur via the enzymic subsites, with the sialic acid mimicking the ribosyl phosphate backbone of RNA (Figure 6). Because gangliosides are prevalent on the surface of tumor cells, an affinity for sialic acid residues could explain the favorable therapeutic index of onconase.

After localization to a cell-surface ganglioside, a ribonuclease is likely to undergo internalization into an endosome. Much of the enzyme is likely to suffer degradation in the lysosome. Nonetheless, some of the ribonuclease could be transported in a retrograde manner to the Golgi apparatus and endoplasmic reticulum. Gaining access to RNA must involve crossing a cellular membrane. Translocation into the cytosol would then enable the ribonuclease to encounter RNA. This putative mechanism is shown in Figure 11.

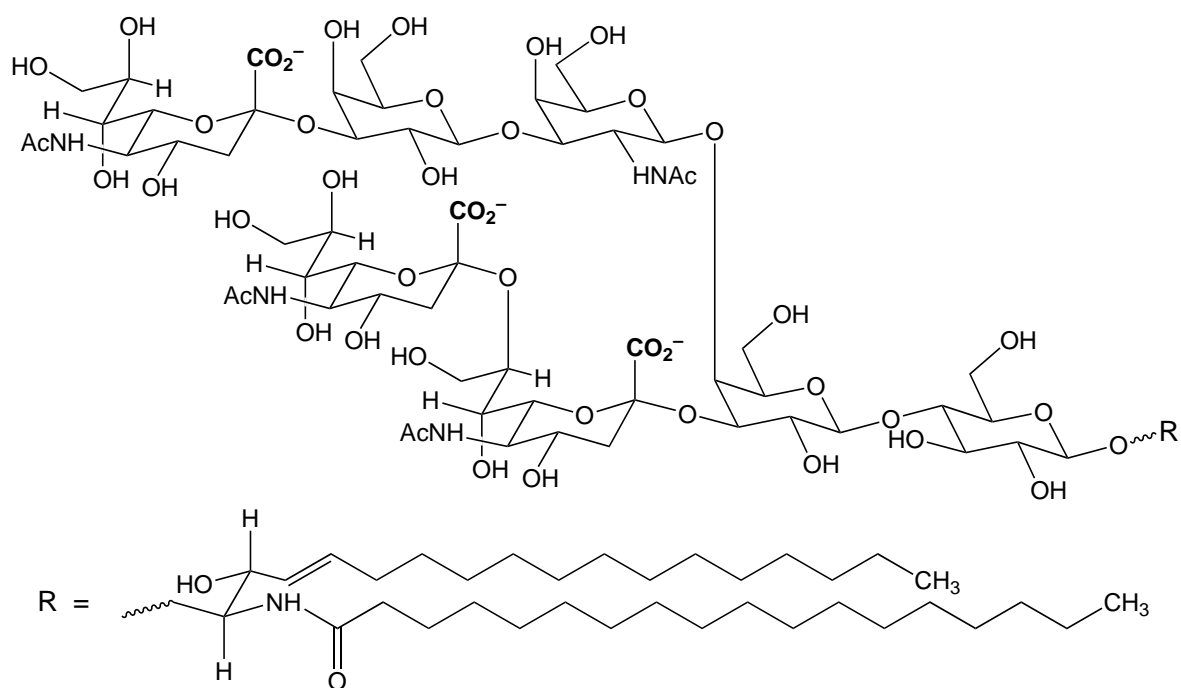


Figure 10. Structure of ganglioside GT1b, a cell-surface glycolipid that contains three sialic acid residues and could localize ribonucleases to tumor cells.

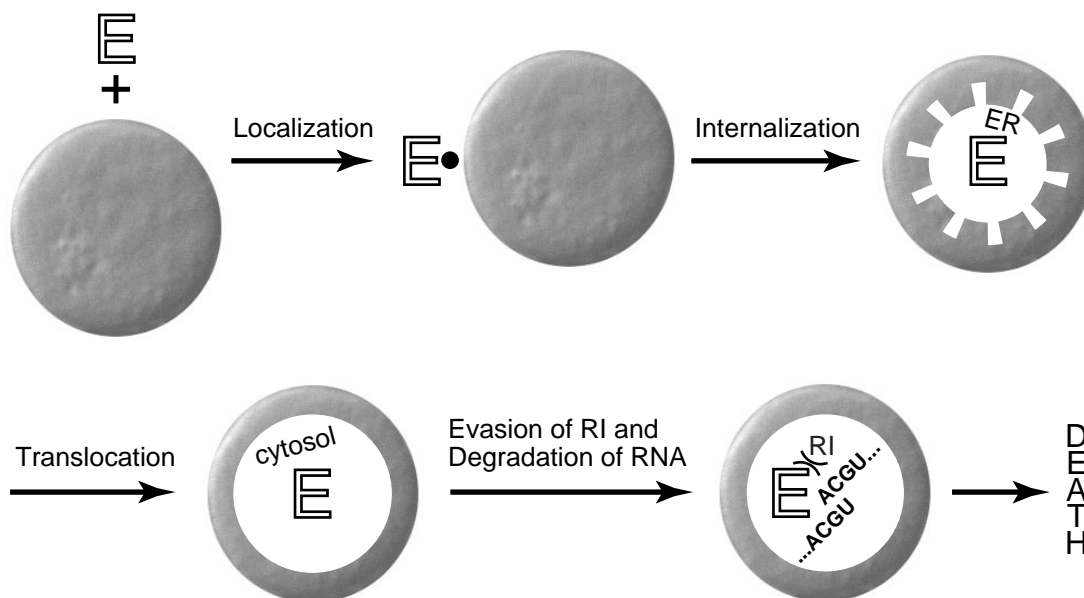


Figure 11. Putative mechanism of ribonuclease cytotoxicity. “E” is a cytotoxic ribonuclease. Localization could involve cell-surface gangliosides (Figure 10).

8. Prospectus

Elaborating the mechanism of ribonuclease cytotoxicity is a fertile area for research at the chemistry – biology interface. Practical consequences of this basic research are apparent. For example, one possibility is to use paradigms from cell biology to increase the flux of ribonuclease molecules into the cytosol. Such an increased flux would enable a ribonuclease to overcome the limitation of a low toxicity number. Another possibility is to engineer a family of ribonucleases directed towards particular types of tissue [25]. Finally, the knowledge gained from the elaboration of the mechanism of ribonuclease cytotoxicity is likely to provide insight into the mechanism of action of angiogenin, an RNase A homolog that promotes neovascularization [26].

Acknowledgment

This paper is dedicated to my colleague, Mo Cleland, to mark the thirty-fifth anniversary of the publication of his landmark papers on the steady-state kinetics of multisubstrate enzymes in *Biochimica Biophysica Acta* **67** (1963) 104–137, 173–187, and 188–196. I am grateful to M.C. Hebert and P.A. Leland for comments on this manuscript. Work on ribonuclease A and its homologs in the Raines laboratory is supported by the National Institutes of Health.

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