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Bovine Pancreatic Ribonuclease: Fifty Years of the First Enzymatic Reaction Mechanism

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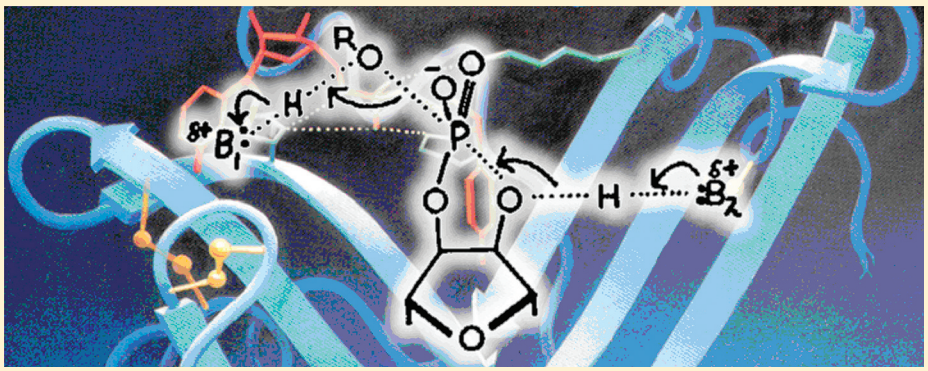

ABSTRACT: Fifty years ago, the group of Tony Mathias and Bob Rabin at University College London deduced the first mechanism for catalysis by an enzyme, ribonuclease [Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R., and Ross, C. A. (1961) *Nature 190*, 781−784]. Here, we celebrate this historic accomplishment by surveying knowledge of enzymology and protein science at that time, facts that led to the formulation of the mechanism, criticisms and alternative mechanisms, data that supported the proposed mechanism, and some of the refinements that have since provided a more precise picture of catalysis of RNA cleavage by ribonucleases. The Mathias and Rabin mechanism has appeared in numerous textbooks, monographs, and reviews and continues to have a profound impact on biochemistry.

The enzymatic reaction mechanism of bovine pancreatic ribonuclease (RNase A) was put forward in 1961, 50 years ago, by the group of Bob Rabin and (the late) Tony Mathias at University College London. In a paper to *Nature*,[1] they presented a summary of the experimental work that was described in detail a year later.[2−6] Their catalytic mechanism was the first in which clear roles were assigned to functional groups of a protein. Other mechanisms followed soon, such as those of chymotrypsin[7] and lysozyme.[8] Although some refinements have been added to the original mechanism, its basic characteristics are still accepted, 50 years after its publication, as those that represent best the catalytic process. In this work, we recall the basic experiments that allowed its formulation, the criticisms that were raised, the alternative mechanisms put forward, and the refinements added later.

It is of the greatest historical significance that the experimental tools at hand in 1961 were, by the standards of 2011, quite unsophisticated. For example, it was not until 1963 that the complete sequence of RNase A was reported.[9] Nothing was known about the three-dimensional structure of the protein, which was not determined until 1967 at low resolution,[10] and 1969 at high resolution.[11] As to studies using nuclear magnetic resonance (NMR) spectroscopy, the first to be reported were those of the group of Roberts and Jardetzky in 1969,[12] experiments conducted at only 100 MHz. Recombinant DNA techniques were not available until the 1980s. Thus, only their chemical modification implicated amino acid residues in catalytic events.

WHY STUDY RIBONUCLEASE?

RNase A was one of the first enzymes that could be attained in large amounts in a purified, crystalline form.[13] In addition, the Armour Company prepared a kilogram lot of the enzyme that was distributed among different laboratories to promote an improved knowledge of protein molecules.[14] That availability, added to some of the properties of the enzyme, such as its stability, its lack of a cofactor, and its small molecular size, favored RNase A as a protein of choice for a large number of studies that covered virtually all fields of protein research (for a review, see ref 15).

For enzymologists, however, there existed an important impediment to studying RNase A, the extreme complexity of its substrate, RNA. It was impossible to take measurements that could be subjected to rational kinetic analysis with such a vaguely defined substrate. Markham and Smith had, however,

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demonstrated that both the alkaline- and RNase A-catalyzed breakdown of RNA took place in two steps. In the first step, a 2′,3′-cyclic nucleotide was formed; in the second step, this cyclic nucleotide was hydrolyzed to a 3′-nucleotide by RNase A and to a mixture of 2′- and 3′-nucleotides by alkali.[16] The synthesis of reasonably pure cytidine 2′,3′-cyclic nucleotide[17] allowed the development of an assay method for RNase A that yielded precisely quantifiable data.[18] This assay was the basis for the kinetic experiments used to derive the mechanism.

SOME RELEVANT FACTS KNOWN AT THE TIME

With respect to the catalytic mechanism of RNase A proposed by the group of Mathias and Rabin, the most relevant facts known in 1961 were as follows. (1) The breakdown of RNA catalyzed by RNase A took place, as noted above,[16] in two steps, the first being a transphosphorylation step in which a 2′,3′-cyclic nucleotide was formed at the 3′-terminus of one product and a 5′-hydroxyl group appeared at the 5′-terminus of the other product. In the second step, the 2′,3′-cyclic nucleotide was hydrolyzed to a 3′-nucleotide (Figure 1). The first step was

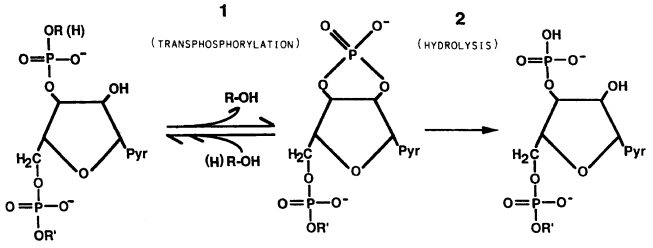


Figure 1. Transphosphorylation and hydrolysis reactions catalyzed by RNase A. Reproduced with permission from ref 53. Copyright 1993 Elsevier.

reversible, whereas the second was practically irreversible. In addition, the second step of the reaction was, formally, the reverse of the first (see below). (2) The RNase A sequence was known in part because of studies by the group of Stein and Moore that, incidentally, led to the development of the automated amino acid analyzer. (3) The chemical modification studies of Barnard and Stein identified His119 as the residue reacting with bromoacetic acid at pH values near neutrality that was necessary for the catalytic activity of RNase A.[19] (4) RNase A was known to have a preference for cleaving phosphodiester bonds in RNA on the 3′-side of pyrimidine residues.[20]

WORK THAT LED TO THE MECHANISM

In 1962, the group of Mathias and Rabin reported the detailed experiments that led to the formulation of the ribonuclease mechanism in a series of five papers in *Biochemical Journal*.[2−6] The papers were numbered 3−7 because they followed two papers published in 1960.[17,18] In the first of the 1962 papers,[2] the group studied the kinetics of RNase A, using cytidine 2′,3′-cyclic phosphate as a substrate, as a function of pH. The results, of the type shown in Figure 2A were interpreted using a kinetic scheme (Figure 2B) that allowed derivation of acid dissociation constants (Figure 2C). The data showed that the pK_a values of two functional groups increased upon binding to the substrate and that the pK_a values of these groups were consistent with their being the imidazoyl groups of histidine side chains.

In the second and third papers[3,4] of the 1962 series, the group of Mathias and Rabin studied the effect of organic solvents on the kinetics of the reaction, again using cytidine 2′,3′-cyclic phosphate as a substrate. The results (Figure 3) showed that the acidic group involved in catalysis was a cationic acid of the AH^+ type (like an imidazolium ion) and not a neutral acid of the AH type. In addition, they showed that methanolysis of the substrate catalyzed by the enzyme (which is the formal reverse of the first step of the reaction) was much faster than hydrolysis, concluding that the 5′-side of the substrate had to interact in some way with the enzyme.

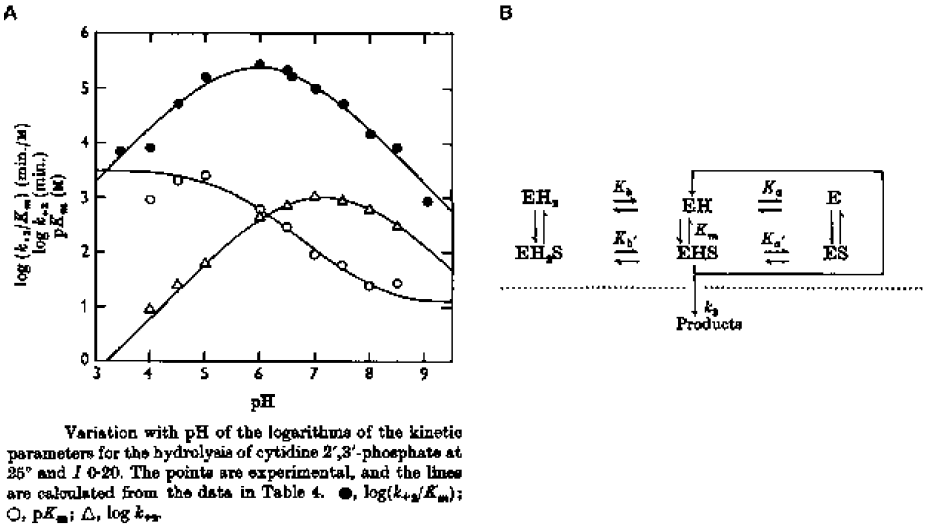
In the fourth paper of that series,[5] the group of Mathias and Rabin studied the binding of Zn^{2+} to RNase A, as well as the efficacy of several cytidine nucleotides as competitive inhibitors of catalysis. They showed that the most efficient inhibitor was cytidine 2′-phosphate, inferring that a specific interaction existed between the 2′-oxygen of the substrate and the enzyme, that the interaction with the 3′-oxygen likely occurred through a water molecule, and that a histidine residue was involved in these interactions.

All of these facts were put together in the last paper of the series,[6] in which a mechanism involving general acid−base catalysis promoted by two histidine residues was put forth. The path of the electrons and the transition state involved in the hydrolysis reaction is depicted in Figure 4. In this representation, **I** is the imidazole that binds either water or alcohol and **II** is the imidazolium ion that interacts with the 2′-oxygen atom of the substrate. The group of Mathias and Rabin also presented a model of the enzyme·substrate complex (Figure 5) in which **I** and **II** are the same imidazoles as in Figure 4, **III** represents the sites of additional interaction with the alcohol, and **IV** is the specificity site in which N-1 of the pyrimidine nucleobase would seem to interact with the enzyme. A hydrogen bond between imidazolium ion **II** and O-2′ weakens the P−O-2′ bond and renders the phosphorus more susceptible to nucleophilic attack; a hydrogen bond between imidazole **I** and a nucleophilic alcohol increases the nucleophilicity of its oxygen. The roles of **I** and **II** are reversed in the transphosphorylation reaction (Figure 1, first step).

CRITICISMS AND ALTERNATIVE MECHANISMS

The Mathias and Rabin mechanism for catalysis by RNase A was, at the beginning, questioned in several respects. On one hand, it was known that rate increases that could be attained by either general acid or general base catalysis could not alone explain the high catalytic efficiency of the enzyme. On the other, the pK_a values obtained for the histidines (Figure 2C) were somewhat divergent from that of imidazole, either free or in the side chain of the amino acid. It should be noted that in 1961, the enormous increases in rate that could be attained from binding energy were not known.[21] Neither were known the large perturbations to the pK_a values of a functional group that can occur in an enzymic active site.[22,23]

Among the alternative mechanisms that have been suggested, those of Witzel,[24] Wang,[25] and Breslow[26] were the most relevant. In the Witzel mechanism, only one histidine was involved, the role of the other being taken by O-2 of the pyrimidine nucleobase. The Wang mechanism invoked a charge-relay system analogous to that of chymotrypsin, and in the mechanism proposed by Breslow, a phosphorane intermediate was involved, imposing a second transition state on the path from substrate to product.



Variation with pH of the logarithms of the kinetic parameters for the hydrolysis of cytidine 2′,3′-phosphate at 25° and *I* 0·20. The points are experimental, and the lines are calculated from the data in Table 4. ●, $\log(k_{+2}/K_m)$; ○, pK_m; △, $\log k_{+2}$.

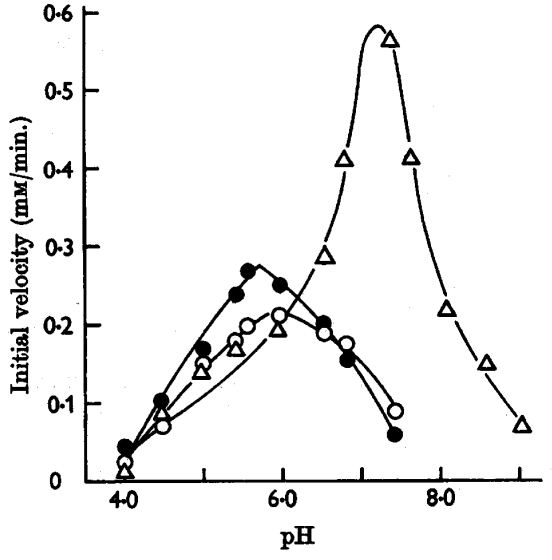
C

Dissociation constants and other parameters for the variation of the kinetics with pH

All measurements were made at 25° and at *I* 0·20.

	Free enzyme		Enzyme–substrate complex
K_b	$(5{\cdot}97 \pm 2{\cdot}74) \times 10^{-8}$	$K_{b'}$	$(5{\cdot}04 \pm 1{\cdot}03) \times 10^{-7}$
K_a	$(1{\cdot}68 \pm 0{\cdot}77) \times 10^{-7}$	$K_{a'}$	$(7{\cdot}90 \pm 1{\cdot}62) \times 10^{-6}$
pK_b	5·22±0·20	$pK_{b'}$	6·30±0·09
pK_a	6·78±0·20	$pK_{a'}$	8·10±0·09
k_{+2}/K_m	$(3{\cdot}2 \pm 1{\cdot}5) \times 10^{8}$ min./M	k_{+2}	1240±250 min.

Figure 2. pH–rate profiles for catalysis of the hydrolysis of cytidine 2′,3′-cyclic phosphate by RNase A. (A) Data. (B) Interpretative scheme. (C) Derived parameters. Reproduced with permission from ref 2. Copyright 1962 Portland Press.



pH–activity curves for ribonuclease in a series of neutral-acid buffers: in water (△), in 50% (v/v) dioxan (●), and in 50% (v/v) formamide (○). The concn. of substrate was 0·05M, and that of ribonuclease 0·0125 mg./ml.

Figure 3. Solvent dependence of the pH–rate profiles for catalysis of the hydrolysis of cytidine 2′,3′-cyclic phosphate by RNase A. Reproduced with permission from ref 4. Copyright 1962 Portland Press.

SUPPORT FOR THE MATHIAS AND RABIN MECHANISM

Several lines of evidence provided support to the mechanism of Mathias and Rabin. Experiments with haloacetic acids confirmed the important role of both His12 and His119,[27,28] and although

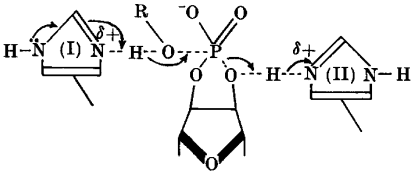


Figure 4. Mechanism of Mathias and Rabin for catalysis of the hydrolysis (R = H) of cytidine 2′,3′-cyclic phosphate by RNase A. Reproduced with permission from ref 6. Copyright 1962 Portland Press.

the pK_a values so obtained were somewhat different from those obtained by kinetic studies, they were close enough to identify them as the groups involved in general acid–base catalysis. The high-resolution three-dimensional structure of RNase S[11] showed that histidines 12 and 119 were at the right distance to interact in a manner consistent with the proposed transition state (Figure 4). In contrast, O-2 of the pyrimidine nucleobase was too distal to interact with the substrate. The results of ^{1}H NMR spectroscopy experiments[12] were also in agreement with the Mathias and Rabin mechanism. The studies on the stereospecificity of the reaction conducted by Eckstein and co-workers,[29] using chiral phosphorothioate analogues of the substrate, demonstrated that the reaction followed an in-line mechanism as opposed to an adjacent one. These conditions were fulfilled by the Mathias and Rabin mechanism and not by that of Witzel. Further confirmation came from molecular dynamics studies by Deakyne and Allen[30] that could be explained only in terms of the proposed mechanism. Finally, the results of studies using recombinant DNA technology to create variants at His12 and His119[31−33] and measuring heavy atom isotope effects on catalysis[34,35] were in full accord with a concerted mechanism that employs these residues in general acid–base catalysis.

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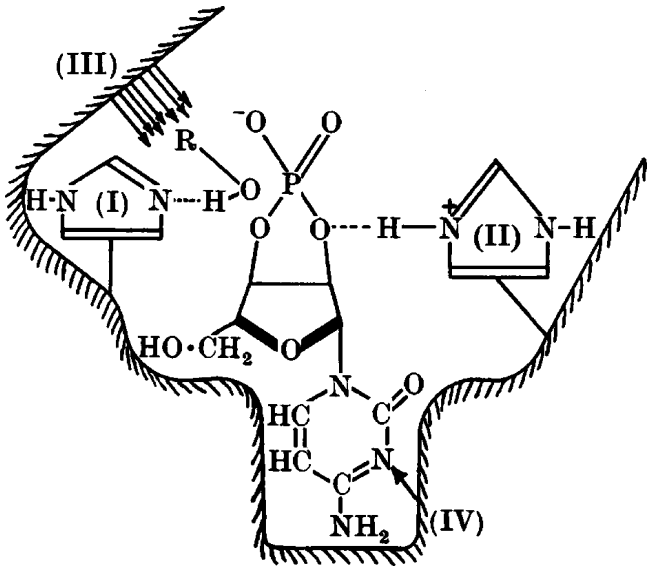
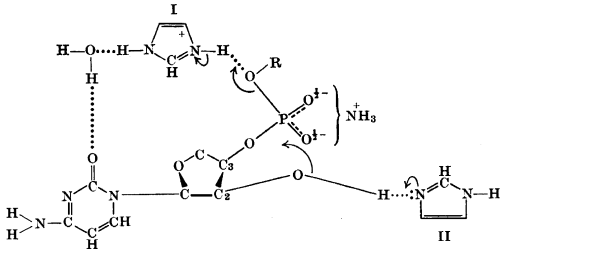


Figure 5. Model of the RNase A·substrate complex. The substrate here is cytidine 2′,3′-cyclic phosphate. **I** and **II** are the imidazolyl groups of two histidine residues. **III** is an enzymic region that interacts with an alcohol or water. **IV** is the specificity region of the enzyme that interacts with the pyrimidine nucleobase of the substrate. Reproduced with permission from ref 6. Copyright 1962 Portland Press.

REFINEMENTS OF THE MECHANISM

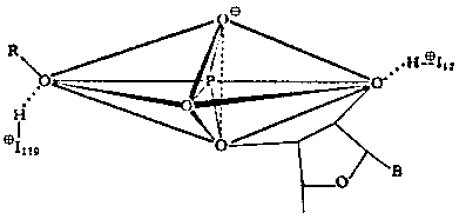
In 1966, the same team of Mathias and Rabin provided a more refined structure of the transition state.[36] An additional cationic acid, such as the ammonium group in the side chain of a lysine residue, was proposed to stabilize the negative charge on the nonbridging oxygens of the phosphoryl group (Figure 6), an



Enzyme–substrate complex of ribonuclease and an ester of cytidine 3′-phosphate. In the model, imidazole II lies between the purine ring of R and the cytosine ring.

Figure 6. Refined model of the RNase A-substrate complex showing the interaction of an ammonium group with nonbridging oxygens of the phosphoryl group. Reproduced with permission from ref 36. Copyright 1966 Portland Press.

aspect confirmed in 1995 by site-directed mutagenesis.[37] Later,[38] they applied the criteria established in phosphorus chemistry[39] and postulated that the transition state was a trigonal bipyramid (Figure 7). NMR spectroscopy allowed the precise determination of the pK_a values of the four histidine residues in RNase A and showed that the pK_a values of His12 and His119, both in the free enzyme and in the presence of a substrate analogue, were in agreement with the data obtained with kinetic methods.[40] Eventually, the pK_a values assigned initially to His12 and His119 had to be exchanged.[41,42] Nonetheless, this reassignment had no effect on the rationale for the catalytic mechanism.



Suggested trigonal bipyramid intermediate formed between enzyme and substrate during catalysis by ribonuclease.

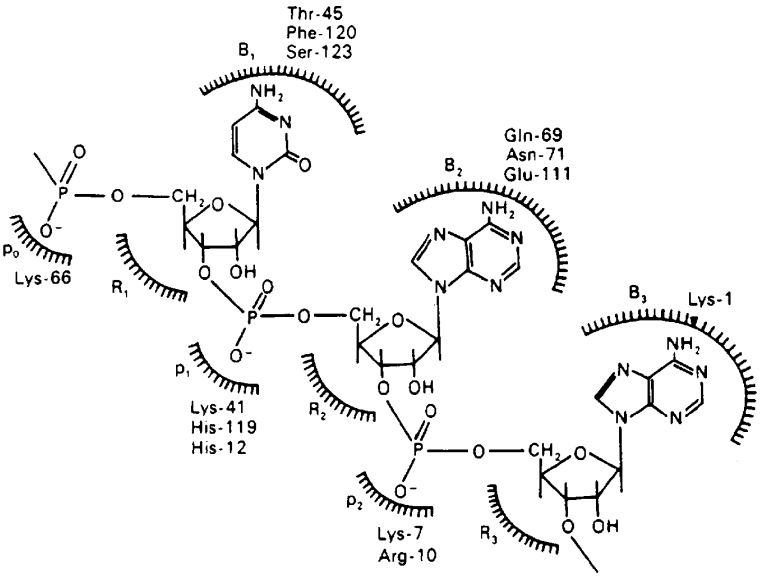
Figure 7. Trigonal bipyramid formed during catalysis by RNase A. Reproduced with permission from ref 38. Copyright 1969 Academic Press.

One of us (C.M.C.) started to work with Tony Mathias and Bob Rabin at University College London in 1966. The goal was to determine the identity of the residues in region **III** (Figure 5), as it was known that the type of nucleobase (R in Figure 5) had an important effect on the rate of catalysis for RNA cleavage. For example, the cleavage rate for substrates of the type NpN′, where N is a pyrimidine, was known to depend on N′ and to decrease in the following order: A > G > C > U.[43] The electrophile 6-chloropurine was used to label region **III** and led to the identification of several enzymic amino groups proximal to the active site.[38] A more elaborate affinity label, the 5′-nucleotide of 6-chloropurine, reacted specifically with the α-amino group of Lys1.[44] Stereochemical and other considerations allowed us to postulate the presence of a secondary site (p_2) that bound the phosphoryl group on the 3′-side of the scissile bond. Similarly, other subsites were postulated, and eventually, some of them were assigned to distinct residues in RNase A.[45] Thus, a great deal of information is now available about region **III** in Figure 5. A scheme of the amino acid residues involved in each subsite is shown in Figure 8. A fourth phosphate-binding subsite was identified in 1998.[46] In assays at low salt concentrations, the favorable Coulombic interactions engendered by these phosphate-binding subsites lead to a second-order rate constant (k_{cat}/K_M = 2.7 × 10^9 M^{-1} s^{-1}[47]) that is among the largest in enzymology.

Studies on the RNase A subsites have provided insight into the unusual kinetic behavior for the turnover of either the mononucleotide cytidine 2′,3′-cyclic phosphate at very high concentrations or cytidine oligonucleotides of various lengths.[48] They have also provided an explanation for the exo- and endonucleolytic activities of RNase A with poly(C) as a substrate.[49] Finally, it has been possible to construct an alternative active site by replacing the constituent amino acids (Lys7 and Arg8) of the p_2 subsite with two histidines that act like His12 and His119 of the original active site located in the p_1 subsite.[50]

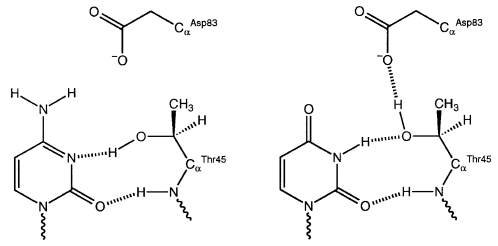
In region **IV** of Figure 5, the side chain hydroxyl and main chain carbonyl groups of Thr45 mediate the specificity of RNase A by forming hydrogen bonds with a pyrimidine nucleobase and excluding a purine nucleobase.[51] Asp83 also affects the uridilyl specificity of the enzyme through an interaction that relies on the side chain of Thr45 forming a hydrogen bond with the side chain of Asp83 (Figure 9). The space created by replacing Thr45 with a smaller alanine or glycine residue allows RNase A to cleave poly(A) efficiently and processively.[52]

A final aspect of the enzymatic reaction is now clear. The traditional depiction has the reaction of RNase A taking place in two steps: transphosphorylation with the formation of a 2′,3′-cyclic phosphate followed by hydrolysis of the cyclic nucleotide



Schematic diagram of the active center cleft in the RNase A-substrate complex. *B*, *R*, and *p* indicate binding subsites for base, ribose, and phosphate, respectively. B_1 is specific for pyrimidines, and B_2 prefers purines. 3′-Pyrimidine mononucleotides bind to $B_1R_1p_1$. The phosphate group of the phosphodiester bond broken by the enzyme binds to p_1.

Figure 8. Subsites of RNase A. B_n, R_n, and p_n are nucleobase-, ribose-, and phosphate-binding subsites, respectively, and are shown with their constituent residues. Reproduced with permission from ref 48. Copyright 1998 American Society for Biochemistry and Molecular Biology.



Hydrogen bonds formed between a bound cytidine (left) or uridine (right) nucleotide and the residues of the B1 subsite of RNase A (W. A. Gilbert, A. L. Fink & G. A. Petsko, unpublished results; Wlodawer *et al.*, 1983).

Figure 9. Hydrogen bonds formed by pyrimidine nucleobases and residues in the B_1 subsite of RNase A. Reproduced with permission from ref 51. Copyright 1995 Elsevier.

to a 3′-nucleotide (Figure 1). Do the two reactions take place sequentially, with the intermediate 2′,3′-cyclic phosphate remaining bound to the enzyme? We showed, separately,[53,54] that this depiction is incorrect; RNase A is actually an "RNA depolymerase".[55] For example, during the breakdown of poly(C) by RNase A, 2′,3′-cyclic phosphates of a progressively lower molecular mass accumulate in the medium and undergo hydrolysis only when no susceptible phosphodiester bonds remain intact.[53] These accumulating intermediates are readily apparent with ^{31}P NMR spectroscopy.[54] The hydrolysis step, which is much slower than the transphosphorylation step, is essentially the microscopic reverse of the transphosphorylation step in which R in Figure 5 is simply a hydrogen atom. This added precision does not contravene the mechanism of Mathias and Rabin, as they had considered that the reactions could be treated as the reverse of each other and that the histidine residues took on opposite roles in each reaction.[6] After each catalytic cycle, the proper form, protonated or unprotonated, of His12 and His119 has to be reset, probably through rapid proton exchange reactions mediated by water or buffer ions.

CONTRIBUTION OF THE MECHANISM TO BIOCHEMISTRY

The mechanism that we have been discussing is, at present, acknowledged as the one that fits the known data on RNase A most faithfully. Inevitably, more details will be reported, but these will not affect the basis of the mechanism. They will only add precision to what is accepted as an accurate picture on how RNase A modifies its RNA substrate. The importance of this mechanism can be seen in the number of general reviews that have been published regularly on RNase A and that consider it to be the most plausible. Among them we can mention those of Richards and Wyckoff in 1971,[15] Blackburn and Moore in 1982,[56] Eftink and Biltonen in 1987,[57] Lolis and Petsko in 1990,[58] D'Alessio and Riordan in 1997,[59] and Raines in 1998.[60] Several specialized books on enzymology also include this mechanism.[61−65] In addition, we note that the mechanism has been depicted in many of the most influential textbooks of biochemistry.[66−70]

In summary, the first catalytic mechanism of an enzyme was deduced 50 years ago by an extraordinary integration of attainable knowledge about enzymology and protein chemistry.[1] This mechanism is accepted widely today. On this notable anniversary,[2] we want to pay due homage to Tony Mathias, Bob Rabin, and their co-workers, who contributed so consequentially to the field of mechanistic enzymology.

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Notes

[a]We note that the deduction of the first enzymatic reaction mechanism in 1961 coincides with the founding of the journal *Biochemistry*.

[b]The k_{cat}/K_M curve (●) in Figure 2A has become a canonical "bell-shaped" pH−rate profile.

■ ABBREVIATIONS

poly(A), poly(adenylic acid); poly(C), poly(cytidylic acid); RNase, ribonuclease.

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