

# Active Site of Ribonuclease A

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## 1 Introduction

Ribonuclease A (RNase A; EC 3.1.27.5) was perhaps the most studied enzyme of the 20th century and is the best characterized ribonuclease. The “A” in its name refers not to its substrate specificity, but to the predominant form of the enzyme produced by the bovine pancreas. RNase A is unmodified, whereas RNase B, RNase C, and RNase D are mixtures of glycoforms. Because of its availability in large quantity and high purity, RNase A has been the object of landmark work in protein chemistry and enzymology (see Cuchillo et al. 1997; Raines 1998 and references therein). In addition, cytotoxic RNase A variants and homologues have demonstrated utility as chemotherapeutic agents (Leland and Raines 2001).

Recognition of the seminal role of RNase A in the chemical sciences reached a pinnacle in 1972, when all three Nobel Prizes in chemistry were awarded for work on this enzyme. Stein and Moore were acknowledged “for their contribution to the understanding of the connection between chemical structure and catalytic activity of the active center of the ribonuclease molecule” (Moore and Stein 1973). Anfinsen was recognized “for his work on ribonuclease, especially concerning the connection between the amino acid sequence and the biologically active conformation” (Anfinsen 1973). Another Nobel Prize in chemistry was added in 1984. In that year, Merrifield was acknowledged “for his development of methodology for chemical synthesis on a solid matrix” (Merrifield 1984). He too chose RNase A as his model system, making it the first protein to succumb to total synthesis.

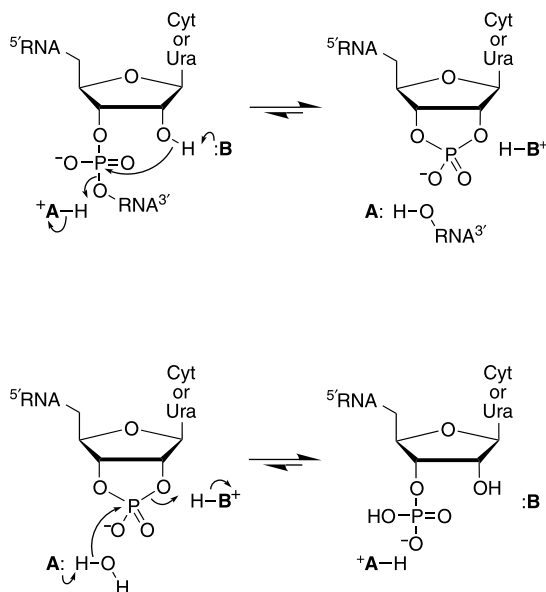
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## 2 Mechanism of Catalysis

RNase A catalyzes the cleavage of the P-O<sup>5'</sup> bond of RNA on the 3' side of cytidine and uridine residues. A mechanism of catalysis that is consistent with all known data from work on the enzyme is depicted in Fig. 1 (Findlay et al. 1961). In this mechanism, the imidazole 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 via a slightly associative transition state to displace a nucleoside (Usher et al. 1970, 1972; Sowa et al. 1997). The imidazolium side chain of His119 acts as an acid that protonates the 5'-oxygen to facilitate its displacement. The two products are then released to solvent. The slow hydrolysis of the nucleoside 2',3'-cyclic phosphate occurs in a separate process that resembles the reverse of transphosphorylation (Cuchillo et al. 1993; Thompson et al. 1994).

The transphosphorylation and hydrolysis reactions catalyzed by RNase A occur via transition states having a pentavalent phosphorus atom. Uridine 2',3'-cyclic vanadate bears some resemblance to this transition state (Lindquist et al. 1973), as well as to the substrate and product of the reaction (Messmore and Raines 2000b). The structure of its crystalline complex with RNase A has provided valuable insight into the catalytic mechanism of RNase A (Wlodawer et al. 1983; Ladner et al. 1997). The active site of this structure is shown in Fig. 2. In the active site, the side chains of His12, His119, Lys41,



**Fig. 1.** Putative mechanism of the transphosphorylation reaction (*top*) and hydrolysis reaction (*bottom*) catalyzed by ribonuclease A. **B** is His12, and **A** is His119. (Findlay et al. 1961)

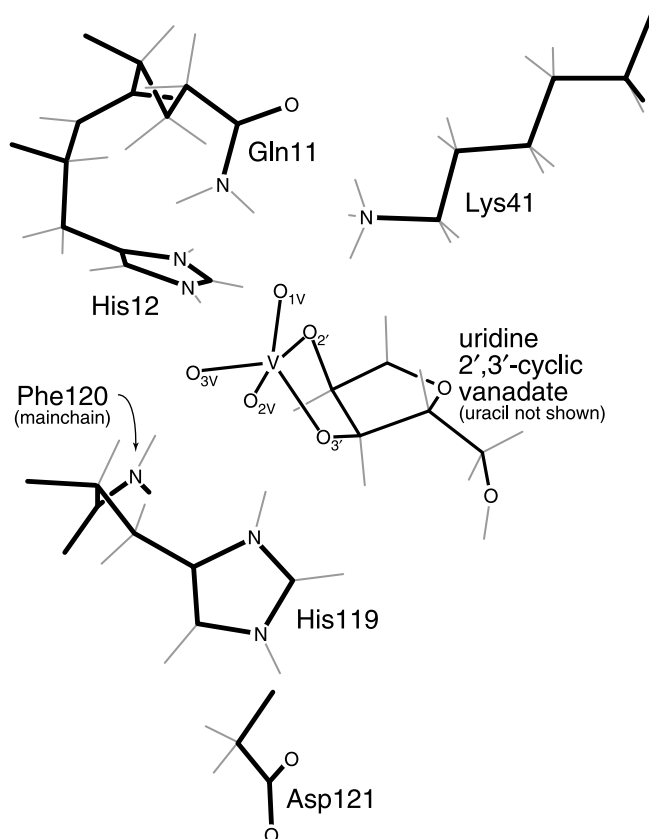


Fig. 2. Crystalline structure of the active site of ribonuclease A bound to uridine 2',3'-cyclic vanadate (Protein Data Bank accession code 1RUV)

Asp121, and Gln11, and the main chain of Phe120 are proximal to the vanadyl group. The apparent roles of these six active-site residues in catalysis are described herein.

### 3 Active-Site Residues

#### 3.1 Histidine 12 and Histidine 119

In early work on catalysis by RNase A, histidines were considered as being critical residues. Specifically, haloacetates were shown both to carboxymethylate its histidine residues and to inactivate the enzyme (Barnard and Stein 1959a, b; Gundlach et al. 1959; Crestfield et al. 1962; Barnard 1969). Under some conditions, only one histidine residue is alkylated in each molecule of

RNase A. The rate of the single carboxymethylation is nearly  $10^4$ -fold greater than that of free histidine and greater than that of carbamoylmethylation of the enzyme, which is consistent with the binding of the anionic form of the haloacetate in a cationic active site. This limited alkylation modifies only His12 or His119.

Catalysis by RNase A has a classic bell-shaped pH-rate profile for both the cleavage of dinucleotide substrates and the hydrolysis of nucleoside 2',3'-cyclic phosphate substrates (Herries et al. 1962; Witzel 1963; del Rosario and Hammes 1969). This profile is consistent with a mechanism that involves two titratable residues, one of which is protonated and the other unprotonated. His12 and His119 are the only residues that need be invoked to explain this pH dependence. Support for this assignment comes from the semisynthesis of a catalytically active RNase A variant containing a 4-fluorohistidine residue at positions 12 and 119 of RNase A (Jackson et al. 1994). The pH-rate profile of this variant retains its bell shape, but shifts to lower pH. Because the  $pK_a$  of 4-fluorohistidine is lower than that of histidine, this perturbation is consistent with both 4-fluorohistidine residues participating in catalysis.

Recombinant DNA techniques have been used to produce RNase A variants in which either His12 or His119 is replaced with an alanine residue (Thompson and Raines 1994). These changes have no significant effect on the three-dimensional structure of the crystalline enzyme (Park et al. 2001). Eliminating the imidazole group of His12 results in a  $10^4$ -fold decrease of the value of  $k_{cat}/K_M$  for cleavage of poly(cytidylic acid) (poly(C)), uridylyl(3',5')adenosine (UpA), and UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. Eliminating the imidazole group of His119 decreases the value of  $k_{cat}/K_M$  by  $10^4$ -fold for the cleavage of poly(C), and by almost  $10^4$ -fold for the cleavage of UpA. In contrast, this change has no significant effect on the value of  $k_{cat}/K_M$  for the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. Thus, the value of the imidazole group of His119 to catalysis depends on the  $pK_a$  of the conjugate acid of the leaving group. The leaving groups in poly(C) and UpA have conjugate acids with  $pK_a \approx 14.8$ , which is the  $pK_a$  of CH<sub>3</sub>OCH<sub>2</sub>CH<sub>2</sub>OH (Ballinger and Long 1960). In contrast, the *p*-nitrophenolate leaving group of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> has a conjugate acid with  $pK_a = 7.14$  (Fickling et al. 1959). Thus, the contribution of His119 to catalysis decreases as the  $pK_a$  of the conjugate acid of the leaving group decreases. This correlation is strong evidence that the role of His119 is to protonate the leaving group during RNA cleavage. Likewise, Brønsted analyses of catalysis by wild-type RNase A (leaving-group  $\beta = -0.19$ ; Davis et al. 1988b) and imidazole (leaving-group  $\beta = -0.59$ ; Davis et al. 1988a) are consistent with general acid catalysis in the enzyme-catalyzed reaction.

The mechanistic role of His12 is not discernable from kinetic data. If His12 does indeed act as a base, then His12 is likely to contribute less to the enzyme-catalyzed cleavage of 2'-deoxy-2'-thio-UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> than to that of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. This expectation exists because the 2'-thiol group has  $pK_a$  8.2 (Dantzman and Kiessling 1996), but a related 2'-hydroxyl group has

$pK_a=12.68$  (Veliky et al. 2001). Surprisingly, RNase A does not appear to catalyze the cleavage of 2'-deoxy-2'-thio-UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> (Dantzman 1998) or 2'-deoxy-2'-thio-UpU (Reese et al. 1994). Among substrates with a 2' hydroxyl group, UpA is cleaved faster by RNase A than is either UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> or UpU (Richards and Wyckoff 1971; Thompson and Raines 1994). Although 2'-deoxy-2'-thio-UpA does bind to the active site of RNase A, the values of  $k_{cat}$  and  $k_{cat}/K_M$  for its cleavage are at least 10<sup>5</sup>-fold lower than are those for the cleavage of UpA (Dantzman 1998). The basis for this discrepancy is unclear. Nonetheless, because His119 has been identified as the acid for the cleavage reaction, it is reasonable to put forth His12 as the base.

The rate enhancements conferred by His12 and His119 are in gratifying agreement with those expected for general acid/base catalysis by these residues. For example, suppose a water molecule were to replace the imidazole that is absent from the H12A and H119A variants. The rate enhancements then derived from the Brønsted equation are

$$\frac{k_{\text{wild-type}}}{k_{\text{H12A}}} = \left( \frac{K_a^{\text{H}_3\text{O}^+}}{K_a^{\text{His12}}} \right)^\beta \quad \text{and} \quad \frac{k_{\text{wild-type}}}{k_{\text{H119A}}} = \left( \frac{K_a^{\text{His119}}}{K_a^{\text{H}_2\text{O}}} \right)^\alpha$$

If  $pK_a^{\text{His12}}=5.8$  and  $pK_a^{\text{His119}}=6.2$  (Markley 1975), and  $pK_a^{\text{H}_2\text{O}}=15.7$ , then the Brønsted equation predicts that general base catalysis provides a rate enhancement of 10<sup>7.5β</sup>-fold and general acid catalysis provides a rate enhancement of 10<sup>9.5α</sup>-fold. Values of α and β tend to be near 0.5 for proton transfers between oxygen and nitrogen (Jencks 1985). Thus, the rate enhancements (ca. 10<sup>4</sup>-fold) predicted with this simple model are similar to those observed in experiments.

His119 has also been replaced by an asparagine residue (Panov et al. 1996). This substitution decreases the value of  $k_{cat}/K_M$  by 10<sup>2</sup>-fold for the cleavage of poly(C) and UpA. Unlike an alanine residue, an asparagine residue can favor formation of a hydrogen bond to the leaving group in the transition state. One interpretation of these data is that such a hydrogen bond enhances the affinity of the enzyme for the rate-limiting transition state by 10<sup>2</sup>-fold.

His12 and His119 not only play an important role in catalysis by RNase A, but also contribute to the binding of single-stranded nucleic acids (Park et al. 2001). Isothermal titration calorimetric studies with wild-type RNase A and its H12A and H119A variants show that His12 and His119 contribute 1.4 and 1.1 kcal/mol, respectively, to the stability of the complex with uridine 3'-phosphate (3'-UMP) at pH 6.0. Fluorescence anisotropic studies show that the stability of the complex of wild-type RNase A and 6-carboxyfluorescein-d(AUAA) increases by 2.4 kcal/mol as the pH decreases from 8.0 to 4.0, and His12 and His119 become protonated. At pH 4.0, replacing His12 with an alanine residue decreases the stability of the complex with 6-carboxyfluorescein-d(AUAA) by 2.3 kcal/mol.

### 3.2. Lysine 41

Early chemical modification work suggested that Lys41 contributes to the catalytic activity of RNase A (Murdock et al. 1966). This proposition was supported by the finding that a variant in which Lys41 is replaced by an arginine residue has only 2 % of the activity of the wild-type enzyme for the hydrolysis of cytidine 2',3'-cyclic phosphate (Trautwein et al. 1991).

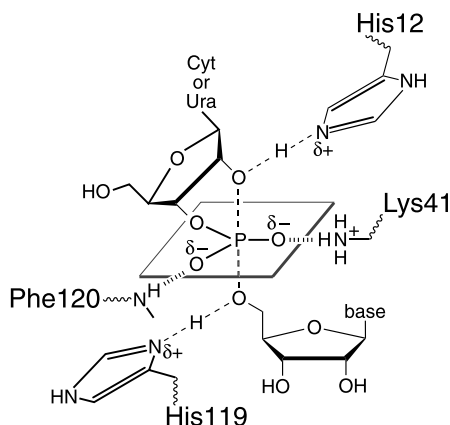
Cysteine elaboration has been used to reveal the role of Lys41 in catalysis (Messmore et al. 1995). Specifically, Lys41 was replaced with a cysteine residue, which was then alkylated with five different haloalkylamines. In the resulting enzymes, high values of  $k_{\text{cat}}/K_M$  for poly(C) cleavage correlate with low values of  $\text{p}K_a$  for the side chain. Moreover, a side chain with an amidinium group (that is,  $-\text{C}(=\text{NH}_2)\text{NH}_2^+$ ), which can form a second hydrogen bond, does not enhance catalysis. A side chain with a quaternary ammonium group (that is,  $-\text{N}(\text{CH}_3)_3^+$ ), which is cationic but cannot be a hydrogen bond donor, gives low activity. These data support a model in which the role of Lys41 is not merely Coulombic. Rather, the role of Lys41 is to form a single hydrogen bond to the transition state during catalysis. Additional studies with these variants at position 41 indicate that Lys41 plays the same role during catalysis of the hydrolysis of uridine 2',3'-cyclic phosphate (Messmore 1999), and contributes to the binding of uridine 2',3'-cyclic vanadate, 3'-UMP, and decavanadate ( $\text{V}_{10}\text{O}_{28}^{6-}$ ) (Messmore and Raines 2000a, b).

The chemical modification of residue 41 enables the creation of a chemical switch for the catalytic activity of RNase A (Messmore et al. 2000). Replacing Lys41 with a cysteine residue results in a  $10^5$ -fold decrease in the value of  $k_{\text{cat}}/K_M$ . Forming a mixed disulfide between the side chain of Cys41 of K41C RNase A and cysteamine reinstalls the amino group and increases the activity by  $10^3$ -fold. This enzyme, which contains a mixed disulfide, is readily deactivated by dithiothreitol. Forming a mixed disulfide between the side chain of Cys41 and mercaptopropyl phosphate, which is designed to place a phosphoryl group in the active site, leads to an additional 25-fold decrease in activity. This enzyme is reactivated in the presence of dithiothreitol and inorganic phosphate, which serves to displace the pendant phosphoryl group from the active site.

### 3.3 Phenylalanine 120

The main-chain nitrogen of Phe120 is proximal to a non-bridging vanadyl oxygen in the complex of RNase A and uridine 2',3'-cyclic vanadate (Fig. 2). Site-directed mutagenesis has been used to replace this residue with glycine, alanine, leucine, glutamate, and tryptophan (Tanimizu et al. 1998; Chatani et al. 2001, 2002). The values of  $k_{\text{cat}}/K_M$  for the cleavage of CpA and the hydrolysis of cytidine 2',3'-cyclic phosphate by these variants are 10- to 100-fold

**Fig. 3.** Putative structure of the chemical transition state during catalysis of RNA cleavage by ribonuclease A. Under optimal conditions, the value of the dissociation constant for this complex is  $K_{TX} \leq 10^{-18}$  M



lower than for wild-type RNase A. The corresponding values of  $k_{cat}$  differ by not more than tenfold. These small effects suggest that the side chain of residue 120 has an indirect role in catalysis. That role appears to be the binding of the pyrimidine nucleobase of the substrate, and the orientation of the side chain of His12 properly for catalysis. In addition, the side chain of Phe120 is important for conformational stability. For example, replacing Phe120 with an alanine residue decreases the conformational stability of RNase A by  $\Delta\Delta G_m = -3.3$  kcal/mol.

To reveal a role for the main-chain nitrogen of Phe120 in catalysis, a non-natural variant of RNase A was constructed using the technique of expressed protein ligation (Evans et al. 1998; Muir et al. 1998). In this non-natural variant, the main-chain nitrogen of Phe120 was replaced with an oxygen, creating an ester linkage. This substitution decreases the value of  $k_{cat}/K_M$  for RNA cleavage by  $10^4$ -fold (R.J. Hondal and R.T. Raines, unpublished results). This large decrease suggests that the main chain of Phe120, like the side chains of His12, His119, and Lys41, makes a critical contribution to catalysis by RNase A. Thus, the transition state for the cleavage of RNA by RNase A is likely to resemble that shown in Fig. 3.

### 3.4 Aspartic Acid 121

In native RNase A, Asp121 is proximal to His119, the acid in the catalysis of RNA cleavage (Fig. 2). The proximity of His119 and Asp121 suggests that these residues form a “catalytic dyad”. In this motif, a histidine residue that mediates general acid/base catalysis forms a hydrogen bond with an aspartate residue, in analogy to the catalytic triad of serine proteases (Schowen 1988; Neurath 1989).

Several attempts have been made to determine the role of the aspartate residue in the His...Asp catalytic dyad of RNase A. In one study, Asp121 was

replaced with asparagine in RNase(1–118)•(111–124), which is a noncovalent complex that resembles RNase A (Lin et al. 1970, 1972). This noncovalent complex consists of residues 1–118 of RNase A (which is obtained by proteolytic digestion of the intact enzyme) and an overlapping synthetic peptide composed of the fourteen C-terminal residues. The D121 N noncovalent complex has ca. 5% of the catalytic activity of RNase(1–118)•(111–124) (Stern and Doscher 1984). These data are difficult to interpret, however, because the three-dimensional structures of RNase(1–118)•(111–124) and its D121 N variant differ significantly (Martin et al. 1987; Cederholm et al. 1991; deMel et al. 1992).

Site-directed mutagenesis of the intact enzyme has been used to replace Asp121 with other residues. The glutamate variant has ca. 17% of the activity of the wild-type enzyme for the hydrolysis of cytidine 2',3'-cyclic phosphate (Trautwein et al. 1991). Replacing Asp121 with an asparagine and alanine residue decreases the values of  $k_{\text{cat}}/K_M$  and  $k_{\text{cat}}$  for the cleavage of UpA and poly(C) by  $10^1$ - and  $10^2$ -fold, respectively (Schultz et al. 1998). The values of  $k_{\text{cat}}/K_M$  and  $k_{\text{cat}}$  for the hydrolysis of U>p are reduced by 3-fold (D121 N) and tenfold (D121A). The alterations do not perturb the three-dimensional structure of the crystalline enzyme, and do not alter the pH-rate profile for hydrolysis. These effects are far less than those observed for analogous variants of serine proteases (Craik et al. 1987; Sprang et al. 1987; Carter and Wells 1988; Sprang et al. 1988; Corey and Craik 1992). Yet, in the structure of D121 N RNase A, N<sub>δ</sub> rather than O<sub>δ</sub> of Asn121 faces His119. Overall, the His...Asp hydrogen bond in the active site of RNase A has a significant but not substantial role in catalysis. This role is likely to position the proper tautomer of His119.

A major difference between Asp121 of RNase A and the aspartate residue in the catalytic triad of serine proteases is solvent exposure – Asp 121 is more accessible to solvent. In native RNase A, Asp121 can form hydrogen bonds with solvent water. It is therefore not surprising that the hydrogen bond in the His...Asp catalytic dyad of RNase A plays a less significant role in catalysis than do the analogous hydrogen bonds in serine proteases.

<sup>1</sup>H-NMR spectra of wild-type RNase A and the D121 N and D121A variants have been analyzed thoroughly as a function of pH (Quirk and Raines 1999). The effect of replacing Asp121 on the microscopic pK<sub>a</sub> values of the histidine residues is surprisingly modest – none change by more than 0.2 units. There is no evidence for the formation of a low-barrier hydrogen bond between His119 and either an aspartate or an asparagine residue at position 121. In the presence of 3'-UMP, protonation of one active-site histidine residue favors protonation of the other. This finding is consistent with the phosphoryl group of 3'-UMP interacting more strongly with the two active-site histidine residues when both are protonated.

A major role of the His...Asp catalytic dyad is to enhance the conformational stability of RNase A. Replacing Asp121 with an asparagine or alanine



residue results in a loss in the conformational stability of  $\Delta\Delta G_m=2.0$  kcal/mol at pH 6.0 (Quirk et al. 1998). This loss is a substantial fraction of the total  $\Delta G_m=9.0$  kcal/mol, and is similar in magnitude to the loss of transition state binding during catalysis of RNA cleavage. The pH-dependencies of the conformational stabilities of the wild-type, D121 N, D121A, and H119A enzymes reveal that the  $pK_a$  of Asp121 is 2.7 in native wild-type RNase A but 3.6 in the denatured enzyme. The side chain of His119 is largely responsible for this change in  $pK_a$ .

### 3.5 Glutamine 11

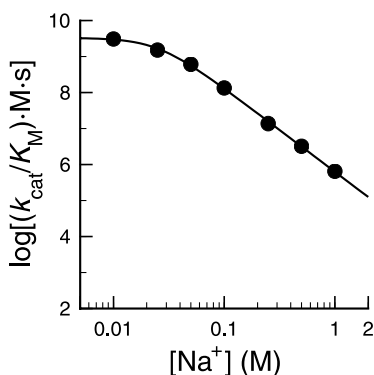
X-ray diffraction analyses show that the side chain of Gln11 can form a hydrogen bond to a substrate, substrate analog, phosphate ion, or sulfate ion bound in the active site of RNase A (Gilliland 1997).  $^1\text{H-NMR}$  spectroscopy provides further evidence for this interaction, as large changes in the  $\text{NH}_{\epsilon_1}$  and  $\text{NH}_{\epsilon_2}$  resonances of Gln11 are observed upon binding of pyrimidine nucleotides (Bruix et al. 1991). In the enzymic complex with uridine 2',3'-cyclic vanadate (Fig. 2), the side-chain nitrogen of Gln11 forms a hydrogen bond with a non-bridging vanadyl oxygen. Work with variants of RNase S, which is the noncovalent complex of S-peptide (residues 1–20) and S-protein (residues 21–124), also ascribes a significant role for Gln11 in catalysis (Marchiori et al. 1974).

The role of Gln11 in catalysis by RNase A has been probed by creating variants in which this residue is replaced with alanine, glutamine acid, and histidine (delCardayré et al. 1995). The results show that, unexpectedly, Gln11 does not lower the free energy barrier for RNA cleavage. Instead, Gln11 serves by increasing the free energy of the enzyme•substrate complex. This increase appears to be due, at least in part, to a binding interaction that reduces non-productive binding. In the absence of the side chain of Gln11, RNase A is more likely to bind an RNA molecule with its phosphoryl group in an improper conformation for in-line attack by the 2'-hydroxyl group. The increase in the number of substrate binding modes causes a decrease in the value of  $k_{\text{cat}}$  and an identical decrease in the value of  $K_M$ , such that the value of  $k_{\text{cat}}/K_M$  is unchanged. This compensation is most dramatic in the turnover of  $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$  by Q11A RNase A. Unlike poly(C) or UpA,  $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$  cannot interact with the subsites of the enzyme on both sides of the scissile bond, making its proper alignment problematic. The values of both  $k_{\text{cat}}$  and  $K_M$  for the cleavage of  $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$  by Q11A RNase A are  $10^2$ -fold lower than are those for the cleavage of  $\text{UpOC}_6\text{H}_4\text{-}p\text{-NO}_2$  by the wild-type enzyme. Thus, a hydrogen bond between the side chain of Gln11 and a phosphoryl oxygen appears to enhance catalysis in a subtle manner – by orienting the substrate so as to prevent it from binding in a nonproductive mode.

## 4 Rate Enhancement

RNase A has only 124 amino acid residues. The molecular formula of the native, uncharged enzyme is  $C_{575}H_{901}N_{171}O_{193}S_{12}$ , which corresponds to a molecular mass of 13,682 Da. How effective is this diminutive enzyme at catalyzing RNA cleavage?

The ratio of  $k_{\text{cat}}/K_M$  for an enzyme-catalyzed reaction to  $k_{\text{uncat}}$  for the uncatalyzed reaction provides a measure of the affinity of the enzyme for the rate-limiting transition state during catalysis (Wolfenden 1976). One complicating factor in applying this measure to RNase A is the dramatic effect of salt concentration on the enzyme-catalyzed reaction. This effect has been examined in detail with enzymatic assays that monitor the cleavage of 6-carboxytetramethylrhodamine-dArU(dA)<sub>2</sub>-6-carboxyfluorescein (6-TAMRA-dArU(dA)<sub>2</sub>-6-FAM), which is a fluorogenic substrate in which a labile ribonucleotide residue is embedded between three deoxynucleotide residues (Kelemen et al. 1999). Unlike shorter substrates, 6-TAMRA-dArU(dA)<sub>2</sub>-6-FAM can interact with all of the known phosphoryl-group binding sites of RNase A. At pH 6.0 and 23 °C, the value of  $k_{\text{cat}}/K_M$  for the cleavage of 6-TAMRA-dArU(dA)<sub>2</sub>-6-FAM is  $6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  in the presence of 1.0 M NaCl (Fig. 4). In the presence of 0.010 M NaCl, this value increases to  $3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , which is among the largest known for an enzyme. At pH 6.0 and 25 °C, the uncatalyzed rate of UpA transphosphorylation, measured by following the cleavage of [5,6-<sup>3</sup>H]Up[3,5,8-<sup>3</sup>H]A for several weeks, is  $5 \times 10^{-9} \text{ s}^{-1}$  (Thompson et al. 1995). The dissociation constant for the rate-limiting transition state during the transphosphorylation of UpA is therefore  $K_{\text{TX}} (=k_{\text{uncat}}/(k_{\text{cat}}/K_M))=10^{-18} \text{ M}$ . Because the rate-limiting transition state may not involve a change in covalency (Thompson et al. 1995; Park and Raines 2002), this value for  $K_{\text{TX}}$  is an upper limit for the dissociation constant of the enzyme bound to the chemical transition state for P-O<sup>5</sup> bond cleavage. At pH 6.0 and 23 °C, the complex of RNase A with the substrate analog 6-FAM-d(AUAA) has  $K_d=8.2 \times 10^{-7} \text{ M}$  in the pres-



**Fig. 4.** Effect of Na<sup>+</sup> concentration on the value of  $k_{\text{cat}}/K_M$  for the cleavage of 6-TAMRA-dArU(dA)<sub>2</sub>-6-FAM by ribonuclease A. (Park and Raines 2001)

ence of 0.018 M NaCl (Fisher et al. 1998). If this complex resembles the Michaelis complex, then RNase A accelerates RNA cleavage by at least  $10^{12}$ -fold.

## 5 Envoi

Its history and simplicity make RNase A an ideal choice for basic research in protein chemistry and enzymology. In addition, RNase A provides chemists with both an inspiration for the design of artificial ribonucleases and a benchmark with which to gauge the success of their designs. The discovery and application of new methods is likely to continue to shed light on the intimacies and intricacies of catalysis by this venerable enzyme.

## References

- Anfinsen CB (1973) Principles that govern the folding of protein chains. *Science* 181:223–230
- Ballinger P, Long FA (1960) Acid ionization constants of alcohols. II. Acidities of some substituted methanols and related compounds. *J Am Chem Soc* 82:795–798
- Barnard EA (1969) Ribonucleases. *Annu Rev Biochem* 38:677–732
- Barnard EA, Stein WD (1959a) The histidine in the active centre of ribonuclease. I. A specific reaction with bromoacetic acid. *J Mol Biol* 1:339–349
- Barnard EA, Stein WD (1959b) The histidine residue in the active centre of ribonuclease. II. The position of this residue in the primary protein chain. *J Mol Biol* 1:350–358
- Bruix M, Rico M, González C, Neira JL, Santoro J, Nieto JL, Rüterjans H (1991) Two dimensional  $^1\text{H-NMR}$  studies of the solution structure of RNase A–pyrimidine-nucleotide complexes. In: de Llorens R, Cuchillo CM, Nogués MV, Parés X (eds) *Structure, mechanism and function of ribonucleases*. Universitat Autònoma de Barcelona, Bellaterra, Spain, pp 15–20
- Carter P, Wells JA (1988) Dissecting the catalytic triad of a serine protease. *Nature* 332:564–568
- Cederholm MT, Stuckey JA, Doscher MS, Lee L (1991) Histidine  $pK_a$  shifts accompanying the inactivating Asp $^{121}$   $\rightarrow$  Asn substitution in a semisynthetic bovine pancreatic ribonuclease. *Proc Natl Acad Sci USA* 88:8116–8120
- Chatani E, Tanimizu N, Ueno H, Hayashi R (2001) Structural and functional changes in bovine pancreatic ribonuclease A by the replacement of Phe120 with other hydrophobic residues. *J Biochem (Tokyo)* 129:917–922
- Chatani E, Hayashi R, Moriyama H, Ueki T (2002) Conformational strictness required for maximum activity and stability of bovine pancreatic ribonuclease A as revealed by crystallographic study of three Phe120 mutants at 1.4 Å resolution. *Protein Sci* 11:72–81
- Corey DR, Craik CS (1992) An investigation into the minimum requirements for peptide hydrolysis by mutation of the catalytic triad of trypsin. *J Am Chem Soc* 114:1784–1790
- Craik CS, Rocznik S, Largman C, Rutter WJ (1987) The catalytic role of the active site aspartic acid in serine proteases. *Science* 237:909–913

- Crestfield AM, Stein WH, Moore S (1962) On the aggregation of bovine pancreatic ribonuclease. *Arch Biochem Biophys Suppl* 1:217–222
- Cuchillo CM, Parés X, Guasch A, Barman T, Travers F, Nogués MV (1993) The role of 2',3'-cyclic phosphodiester in the bovine pancreatic ribonuclease A catalysed cleavage of RNA: Intermediates or products? *FEBS Lett* 333:207–210
- Cuchillo CM, Vilanova M, Nogués MV (1997) Pancreatic ribonucleases. In: D'Alessio G, Riordan JF (eds) *Ribonucleases: structures and functions*. Academic Press, New York, pp 271–304
- Dantzman CL (1998) PhD Thesis, University of Wisconsin–Madison
- Dantzman CL, Kiessling LL (1996) Reactivity of a 2'-thio nucleotide analog. *J Am Chem Soc* 118:11715–11719
- Davis AM, Hall AD, Williams A (1988a) Charge description of base-catalyzed alcoholysis of aryl phosphodiester: A ribonuclease model. *J Am Chem Soc* 110:5105–5108
- Davis AM, Regan AC, Williams A (1988b) Experimental charge measurement at leaving oxygen in the bovine ribonuclease A catalyzed cyclization of uridine 3'-phosphate aryl esters. *Biochemistry* 27:9042–9047
- del Rosario EJ, Hammes GG (1969) Kinetic and equilibrium studies of the ribonuclease-catalyzed hydrolysis of uridine 2',3'-cyclic phosphate. *Biochemistry* 8:1884–1889
- delCardayré SB, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT (1995) Engineering ribonuclease A: production, purification, and characterization of wild-type enzyme and mutants at Gln11. *Protein Eng* 8:261–273
- deMel VSJ, Martin PD, Doscher MS, Edwards BFP (1992) Structural changes that accompany the reduced catalytic efficiency of two semisynthetic ribonuclease analogs. *J Biol Chem* 267:247–256
- Evans TC Jr, Benner J, Xu M-Q (1998) Semisynthesis of cytotoxic proteins using a modified protein splicing element. *Protein Sci* 7:2256–2264
- Fickling MM, Fischer A, Mann BR, Packer J, Vaughan J (1959) Hammett substituent constants for electron-withdrawing substituents: Dissociation of phenols, anilinium ions and dimethylanilinium ions. *J Am Chem Soc* 81:4226–4230
- Findlay D, Herries DG, Mathias AP, Rabin BR, Ross CA (1961) The active site and mechanism of action of bovine pancreatic ribonuclease. *Nature* 190:781–784
- Fisher BM, Ha J-H, Raines RT (1998) Coulombic forces in protein-RNA interactions: Binding and cleavage by ribonuclease A and variants at Lys7, Arg10 and Lys66. *Biochemistry* 37:12121–12132
- Gilliland GL (1997) Crystallographic studies of ribonuclease complexes. In: D'Alessio G, Riordan JF (eds) *Ribonucleases: structures and functions*. Academic Press, New York, pp 305–341
- Gundlach HG, Stein WH, Moore S (1959) The nature of the amino acid residues involved in the inactivation of ribonuclease by iodoacetate. *J Biol Chem* 234:1754–1760
- Herries DG, Mathias AP, Rabin BR (1962) The active site and mechanism of action of bovine pancreatic ribonuclease. 3. The pH-dependence of the kinetic parameters for the hydrolysis of cytidine 2',3'-phosphate. *Biochem J* 85:127–134
- Jackson DY, Burnier J, Quan C, Stanley M, Tom J, Wells JA (1994) A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues. *Science* 266:243–247
- Jencks WP (1985) A primer for the Bema Hapothle. An empirical approach to the characterization of changing transition-state structures. *Chem Rev* 85:511–527
- Kelemen BR, Klink TA, Behlke MA, Eubanks SR, Leland PA, Raines RT (1999) Hypersensitive substrate for ribonucleases. *Nucleic Acids Res* 27:3696–3701
- Ladner JE, Wladkowski BD, Svensson LA, Sjölin L, Gilliland GL (1997) X-Ray structure of a ribonuclease A–uridine vanadate complex at 1.3-Å resolution. *Acta Crystallogr Sect D* 53:290–301

- Leland PA, Raines RT (2001) Cancer chemotherapy – ribonucleases to the rescue. *Chem Biol* 8:405–413
- Lin MC, Gutte B, Moore S, Merrifield RB (1970) Regeneration of activity by mixture of ribonuclease enzymatically degraded from the COOH terminus and a synthetic COOH-terminal tetradecapeptide. *J Biol Chem* 245:5169–5170
- Lin MC, Gutte B, Caldi DG, Moore S, Merrifield RB (1972) Reactivation of des(119–124) ribonuclease A by mixture with synthetic COOH-terminal peptides; the role of phenylalanine-120. *J Biol Chem* 247:4768–4774
- Lindquist RN, Lynn JL Jr, Lienhard GE (1973) Possible transition-state analogs for ribonuclease. The complexes of uridine with oxovanadium(IV) ion and vanadium(V) ion. *J Am Chem Soc* 95:8762–8768
- Marchiori F, Borin G, Moroder L, Rocchi R, Scoffone E (1974) Relation between structure and function in some partially synthetic ribonucleases S'. II. Kinetic determinations on [Orn10, Glu11]-, [Orn10, Leu11]- and [Orn10, Lys11]-RNase S'. *Int J Pept Prot Res* 6:337–345
- Markley JL (1975) Correlation proton magnetic resonance studies at 250 MHz of bovine pancreatic ribonuclease. I. Reinvestigation of the histidine peak assignment. *Biochemistry* 14:3546–3553
- Martin PD, Doscher MS, Edwards BFP (1987) The refined crystal structure of a fully active semisynthetic ribonuclease at 1.8-Å resolution. *J Biol Chem* 262:15930–15938
- Merrifield RB (1984) Solid phase synthesis. *Science* 232:341–347
- Messmore JM (1999) PhD Thesis, University of Wisconsin, Madison
- Messmore JM, Raines RT (2000a) Decavanadate inhibits catalysis by ribonuclease A. *Bioconjug Chem* 11:25–30
- Messmore JM, Raines RT (2000b) Pentavalent organo-vanadates as transition state analogues for phosphoryl transfer reactions. *J Am Chem Soc* 122:9911–9916
- Messmore JM, Fuchs DN, Raines RT (1995) Ribonuclease A: Revealing structure–function relationships with semisynthesis. *J Am Chem Soc* 117:8057–8060
- Messmore JM, Holmgren SK, Grilley JE, Raines RT (2000) Sulfur shuffle: Modulating enzymatic activity by thiol–disulfide interchange. *Bioconjug Chem* 11:408–413
- Moore S, Stein WH (1973) Chemical structures of pancreatic ribonuclease and deoxyribonuclease. *Science* 180:458–464
- Muir TW, Sondhi D, Cole PA (1998) Expressed protein ligation: a general method for protein engineering. *Proc Natl Acad Sci USA* 95:6705–6710
- Murdock AL, Grist KL, Hirs CHW (1966) On the dinitrophenylation of bovine pancreatic ribonuclease A. Kinetics of the reaction in water and 8 M urea. *Arch Biochem Biophys* 114:375–390
- Neurath H (1989) In: Beynon RJ, Bond JS (eds) *Proteolytic enzymes: a practical approach*. IRL Press, New York, pp 1–13
- Panov KI, Kolbanovskaya EY, Okorokov AL, Panova TB, Terwisscha van Scheltinga AC, Karpeisky MY, Beintema JJ (1996) Ribonuclease A mutant His<sup>119</sup>Asn: The role of histidine in catalysis. *FEBS Lett* 398:57–60
- Park C, Raines RT (2001) Quantitative analysis of the effect of salt concentration on enzymatic catalysis. *J Am Chem Soc* 123:11472–11479
- Park C, Raines RT (2003) Catalysis by ribonuclease A is limited by the rate of substrate association. *Biochemistry* 42:3509–3518
- Park C, Schultz LW, Raines RT (2001) Contribution of the active site histidine residues of ribonuclease A to nucleic acid binding. *Biochemistry* 40:4949–4956
- Quirk DJ, Park C, Thompson JE, Raines RT (1998) His<sup>119</sup>Asp catalytic dyad of ribonuclease A: Conformational stability of the wild-type, D121 N, D121A, and H119A enzymes. *Biochemistry* 37:17985–17964

- Quirk DJ, Raines RT (1999) His...Asp catalytic dyad of ribonuclease A: Histidine  $pK_a$  values in the wild-type, D121 N, and D121A enzymes. *Biophys J* 76:1571–1579
- Raines RT (1998) Ribonuclease A. *Chem Rev* 98:1045–1065
- Reese CB, Simons C, Zhang PZ (1994) The synthesis of 2'-thiouridylyl-(3'→5')-uridine. *J Chem Soc, Chem Commun*:1809–1810
- Richards FM, Wyckoff HW (1971) Bovine pancreatic ribonuclease. *The Enzymes* IV:647–806
- Schowen RL (1988) Structural and energetic aspects of proteolytic catalysis by enzymes: Charge-relay catalysis in the function of serine proteases. In: Lieberman JE, Greenberg A (eds) *Mechanistic principles of enzyme activity*. VCH, New York, pp 119–168
- Schultz LW, Quirk DJ, Raines RT (1998) His...Asp catalytic dyad of ribonuclease A: Structure and function of the wild-type, D121 N, and D121A enzymes. *Biochemistry* 37:8886–8898
- Sowa GA, Hengge AC, Cleland WW (1997)  $^{18}O$  isotope effects support a concerted mechanism for ribonuclease A. *J Am Chem Soc* 119:2319–2320
- Sprang S, Standing T, Fletterick RJ, Stroud RM, Finer-Moore JE, Xuong NH, Hamlin R, Rutter WJ, Craik CS (1987) The three-dimensional structure of Asn102 mutant trypsin: Role of Asp102 in serine protease catalysis. *Science* 237:905–909
- Sprang SR, Fletterick RJ, Graf L, Rutter WJ, Craik CS (1988) Studies of specificity and catalysis in trypsin by structural analysis of site-directed mutants. *Crit Rev Biotechnol* 8:225–236
- Stern MS, Doscher MS (1984) Aspartic acid-121 functions at the active site of bovine pancreatic ribonuclease. *FEBS Lett* 171:253–256
- Tanimizu N, Ueno H, Hayashi R (1998) Role of Phe120 in the activity and structure of bovine pancreatic ribonuclease A. *J Biochem* 124:410–416
- Thompson JE, Raines RT (1994) Value of general acid–base catalysis to ribonuclease A. *J Am Chem Soc* 116:5467–5468
- Thompson JE, Kutateladze TG, Schuster MC, Venegas FD, Messmore JM, Raines RT (1995) Limits to catalysis by ribonuclease A. *Bioorg Chem* 23:471–481
- Thompson JE, Venegas FD, Raines RT (1994) Energetics of catalysis by ribonucleases: Fate of the 2',3'-cyclic intermediate. *Biochemistry* 33:7408–7414
- Trautwein K, Holliger P, Stackhouse J, Benner SA (1991) Site-directed mutagenesis of bovine pancreatic ribonuclease: Lysine-41 and aspartate-121. *FEBS Lett* 281:275–277
- Usher DA, Erenrich ES, Eckstein F (1972) Geometry of the first step in the action of ribonuclease-A. *Proc Natl Acad. Sci U.S.A.* 69:115–118
- Usher DA, Richardson DI Jr, Eckstein F (1970) Absolute stereochemistry of the second step of ribonuclease action. *Nature* 228:663–665
- Velikyan I, Acharya S, Trifonova A, Földesi A, Chattopadhyaya J (2001) The  $pK_a$ 's of 2'-hydroxyl group in nucleosides and nucleotides. *J Am Chem Soc* 123:2893–2894
- Witzel H (1963) The function of the pyrimidine base in the ribonuclease reaction. *Progr Nucleic Acid Res* 2:221–258
- Wlodawer A, Miller M, Sjölin L (1983) Active site of RNase: Neutron diffraction study of a complex with uridine vanadate, a transition-state analog. *Proc Natl Acad Sci USA* 80:3628–3631
- Wolfenden R (1976) Transition state analog inhibitors and enzyme catalysis. *Annu Rev Biochem* 5:271–306