

# Ribonuclease A

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Ronald T. Raines was born in 1958 in Montclair, NJ. He received Sc.B. degrees in chemistry and biology from the Massachusetts Institute of Technology. At M.I.T., he worked with Christopher T. Walsh to reveal the reaction mechanisms of pyridoxal 5'-phosphate-dependent enzymes. Raines was a National Institutes of Health predoctoral fellow in the chemistry department at Harvard University. There, he worked with Jeremy R. Knowles to elucidate the reaction energetics of triosephosphate isomerase. Raines was a Helen Hay Whitney postdoctoral fellow in the biochemistry and biophysics department at the University of California, San Francisco. At U.C.S.F., he worked with William J. Rutter to clone, express, and mutate the cDNA that codes for ribonuclease A. Raines then joined the faculty of the biochemistry department at the University of Wisconsin—Madison, where he is now associate professor of biochemistry and chemistry. His honors include the 1998 Pfizer Award in Enzyme Chemistry from the American Chemical Society. His research group uses techniques that span the chemistry–biology interface to reveal protein structure–function relationships *in vitro* and *in vivo*.

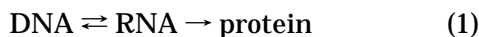
discovery<sup>3</sup> and detailed characterization of bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5). The “A” refers to the predominant form of the enzyme in the pancreas of *Bos taurus*. RNase A is unmodified, whereas RNase B is a mixture of glycoforms in which Man<sub>5–9</sub>GlcNAc<sub>2</sub> is attached to the side-chain nitrogen of Asn34.<sup>4–6</sup> RNase C and RNase D are still less abundant in the bovine pancreas and more heterogeneous in their glycosylation.<sup>7,8</sup>

RNase A has been the object of landmark work on the folding, stability, and chemistry of proteins; in enzymology; and in molecular evolution. Recognition of the historic role of RNase A culminated in 1972 when three researchers were awarded with the Nobel Prize in chemistry for work on this enzyme (Table 1<sup>9–11</sup>). A fourth researcher was honored in 1984.

Researchers continue to choose RNase A as a model system, requiring the frequent compilation of information. Comprehensive books have appeared on nucleases<sup>12,13</sup> and ribonucleases.<sup>14</sup> In addition, au-

## I. Introduction

Biological information is stored by DNA and manifested by proteins. RNA serves as the conduit:<sup>1</sup>



The flow of information through RNA is essential for known life. By catalyzing the synthesis or degradation of RNA, two classes of enzymes control this flow. RNA synthesis is catalyzed by RNA polymerases. RNA degradation is catalyzed by RNA depolymerases, which are most often called “ribonucleases”.

The ribonucleolytic activity in the pancreas of ruminants is particularly high, perhaps to digest the large amount of RNA produced by stomach microorganisms.<sup>2</sup> This high level of activity has led to the

**Table 1. Nobel Prizes in Chemistry for Work on Ribonuclease A**

Nobel laureate	year	Nobel lecture
Christian B. Anfinsen (1916–1996)	1972	“Studies on the principles that govern the folding of protein chains” <sup>9</sup>
Stanford Moore (1913–1982)	1972	“The chemical structures of pancreatic ribonuclease and deoxyribonuclease” <sup>10</sup>
William H. Stein (1911–1980)	1972	“The chemical structures of pancreatic ribonuclease and deoxyribonuclease” <sup>10</sup>
Robert Bruce Merrifield (1921–)	1984	“Solid-phase synthesis” <sup>11</sup>

thoritative reviews on RNase A have disseminated thoughts and information.<sup>15–22</sup> In this review, recent information on the structure and function of RNase A is added to the background of historic work. This review emphasizes applications of recombinant DNA technology and nucleic acid chemistry, which are shedding new light on the chemistry and biology of this venerable enzyme.

## II. Heterologous Production

Changing the residues in a protein and analyzing the consequences of these changes is a powerful method for probing the role of particular functional groups in proteins.<sup>23,24</sup> Although such changes can be made by either total synthesis or semisynthetic procedures, they can be much easier to effect by site-directed mutagenesis of a gene expressed in a heterologous host.

The heterologous production of RNase A has been problematic. The difficulty has been due largely to three obstacles. First, the cDNA of RNase A is difficult to clone because the corresponding RNA must be isolated intact from the pancreas, an organ rich in ribonuclease.<sup>25</sup> Second, RNase A is susceptible to proteolysis when unfolded. Third, high levels of native RNase A are cytotoxic. (See section XII.) These obstacles thwarted the creation of RNase A variants, and work on RNase A began to stall. This lag was made more frustrating by the notable success of early physical and chemical analyses of the enzyme.

The first heterologous system for the expression of RNase A was based on the total synthesis of a gene that codes for RNase A (which followed the total synthesis of a gene that codes for the S-protein fragment<sup>26</sup>) and the expression of that gene in *Escherichia coli* to produce a fusion protein with  $\beta$ -galactosidase.<sup>27</sup> Purifying RNase A from this system was made more efficient by the elimination of the  $\beta$ -galactosidase fusion tag.<sup>28</sup> The RNase A produced had a nonnatural *N*-formyl methionine residue at its *N*-terminus. The more recent addition of a murine signal peptide to this system directed active, mature enzyme to be secreted into the periplasm.<sup>29</sup> This system allows approximately 5 mg of soluble RNase A (and 5 mg of insoluble RNase A) to be recovered from each liter of fermented culture.

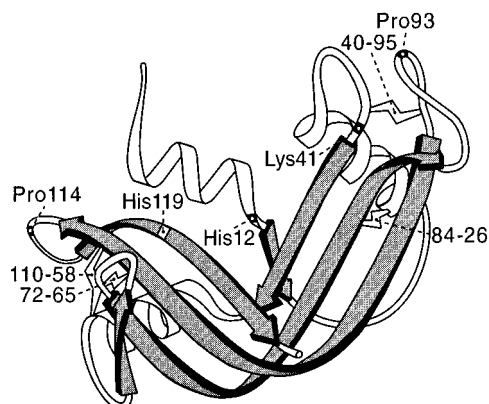
After its synthesis, the gene that codes for RNase A as well as its cDNA were cloned by recombinant DNA methods.<sup>30,31</sup> The DNA sequence that codes for the enzyme itself is preceded by a sequence that codes for a peptide of 26 residues.<sup>30</sup> This peptide begins with a methionine residue, has a basic residue near the amino terminus, is hydrophobic, and terminates with a glycine residue. Each of these features is characteristic of peptides that signal the secretion of proteins. This signal sequence apparently directs the secretion of RNase A from pancreatic exocrine cells.

The cloned gene and cDNA that code for RNase A were expressed initially by relatively low-yielding systems in *E. coli*,<sup>32–34</sup> *Bacillus subtilis*,<sup>35</sup> and *Saccharomyces cerevisiae*.<sup>31,36,37</sup> Similarly, rat pancreatic ribonuclease was produced at low levels in cultured monkey kidney COS-1 cells.<sup>38</sup> RNase 1 (human pancreatic ribonuclease) was produced at low levels in *S. cerevisiae*<sup>39</sup> and in cultured Chinese hamster ovary cells.<sup>40</sup>

Perhaps the most important breakthrough in the heterologous production of RNase A was the development of pET systems.<sup>41</sup> pET systems use the strong T7 RNA polymerase promoter to direct the expression of cloned genes. The resulting proteins are produced in such large quantities that they often aggregate into inclusion bodies. Because RNase A is easy to solubilize and refold, inclusion body formation is not problematic. Rather, the formation of inclusion bodies is beneficial because inclusion bodies are easy to isolate and contain almost pure target protein. Moreover, unfolded RNase A in inclusion bodies lacks ribonucleolytic activity and thus cytotoxicity. By using a pET system, RNase A that is identical to that isolated from bovine pancreas has been produced with isolated yields of ~50 mg per liter of culture.<sup>37</sup> RNase 1 has been produced similarly in *E. coli* cells.<sup>39,42–44</sup> Finally, a new system for the efficient production of active, mature RNase A in the periplasm of *E. coli* cells makes use of the alkaline phosphatase signal peptide and the  $\lambda$  P<sub>R</sub> promoter to produce 40 mg of enzyme per liter of culture.<sup>45,46</sup> The pET and  $\lambda$  P<sub>R</sub> systems now make available virtually unlimited quantities of RNase A in which any amino acid residue is replaced with any other.

## III. Structure

RNase A was first crystallized over 50 years ago,<sup>47,48</sup> and these crystals were shown to diffract to a resolution of 2 Å.<sup>49</sup> RNase A was the first enzyme and third protein (after insulin<sup>50</sup> and hemoglobin<sup>51</sup>) for which a correct amino acid sequence was determined,<sup>52,53</sup> and the third enzyme and fourth protein (after myoglobin,<sup>54,55</sup> lysozyme,<sup>56</sup> and carboxypeptidase A<sup>57</sup>) whose three-dimensional structure was determined by X-ray diffraction analysis.<sup>58</sup> A general method for using fast atom bombardment mass spectrometry (FABMS) to assign completely the disulfide bonds of a protein was developed with RNase A.<sup>59</sup> 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).<sup>60,61</sup> Finally, the use of NMR spectroscopy in elaborating both protein structure<sup>62</sup> and protein folding pathways<sup>63</sup> were developed with RNase A. The <sup>1</sup>H NMR resonances of the enzyme have been assigned, and the structure of the enzyme in solution has been determined.<sup>64–68</sup> NMR spectroscopy



**Figure 1.** Ribbon diagram of the three-dimensional structure of ribonuclease A.<sup>72</sup> The inscriptions refer to the location of the eight cysteine residues that give rise to the four disulfide bonds, the two proline residues with cis peptide bonds, and the three residues most important for catalysis: His12, His119, and Lys41.

copy has also been used to characterize the structure of RNase B.<sup>6,69,70</sup> 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](http://www.pdb.bnl.gov)).

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  $C_{575}H_{907}N_{171}O_{192}S_{12}$ . This formula corresponds to a molecular mass of 13 686 Da. As a small protein, RNase A became a target of synthetic chemists and was the first protein to succumb to total synthesis.<sup>11,71</sup> This preparation had low, but measurable, ribonucleolytic activity.

The overall shape of the enzyme resembles that of a kidney, with the active-site residues lying in the cleft (Figure 1<sup>72</sup>). The predominant elements of secondary structure are a long four-stranded antiparallel  $\beta$ -sheet and three short  $\alpha$ -helices. The enzyme is cross-linked 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 (or *E*) conformation. These proline residues are in type VI<sup>73</sup> reverse turns at opposite ends of the native enzyme.

An important contribution to the understanding of RNase A function has been the determination of the structure of crystalline complexes between the enzyme and nucleic acids that act as substrate or product analogues. Structures with oligonucleotides include those of RNase A with bound  $d(pA)_4$ ,<sup>74,75</sup>  $d(pT)_4$ ,<sup>76</sup> and  $d(ApTpApApG)$ ,<sup>77</sup> and RNase B with bound  $d(pA)_4$ .<sup>78</sup> Structures with dinucleotides include a productive (that is, catalytically meaningful) complex with  $d(CpA)$ ,<sup>79</sup> and unproductive complexes with  $d(CpG)$  and cytidylyl(2'→5')guanosine.<sup>80,81</sup> Structures of RNase A and its complexes, as revealed by X-ray diffraction analysis<sup>82</sup> as well as NMR spectroscopy,<sup>83</sup> have been the subject of recent reviews.

#### IV. Folding and Stability

The stability of RNase A is legendary. The classical procedure for the purification of RNase A from

a bovine pancreas relies on the enzyme maintaining its integrity and solubility under drastic conditions: first, 0.25 N sulfuric acid at 5 °C, and then, pH 3.0 at 95–100 °C.<sup>84</sup> The final step in this protocol calls for crystallization of the enzyme.

The three-dimensional structure of RNase A is fully encoded by its amino acid sequence.<sup>85–89</sup> This discovery made RNase A into a favorite model system for the application of new methods to probe protein folding. In recent examples, electrospray mass spectrometry has been used to determine which disulfide bonds (both native and nonnative) form during the folding of the reduced molecule<sup>90–92</sup> or a derivative in which the eight cysteine residues are in mixed disulfides with glutathione.<sup>92</sup> Fourier transform infrared (FTIR) spectroscopy, with its unique signature for  $\beta$ -sheets, has been used to probe new aspects of RNase A folding.<sup>32,93–95</sup> In these and other studies on the folding of RNase A, the unfolded enzyme is generated by high or low temperature, high or low pH, or chaotropic agents. The unfolding of RNase A by high pressure has attracted much interest, promising still more insights.<sup>96–101</sup>

Two distinct starting materials have been used in most studies on the folding of RNase A: reduced enzyme and oxidized enzyme (with the four native disulfide bonds intact). Studies of the folding of the reduced enzyme have focused on disulfide bond formation. Studies of the folding of the oxidized enzyme have focused on prolyl peptide bond isomerization. These and other aspects of the folding of RNase A have been the subject of a recent review.<sup>102</sup>

#### A. Disulfide Bond Formation

The four disulfide bonds in RNase A are critical to the stability of the native enzyme. Replacing any cystine with a pair of alanine<sup>103</sup> or serine<sup>34,104</sup> residues reduces the thermal stability of the enzyme. The two disulfide bonds (Cys26–Cys84 and Cys58–Cys110) between an  $\alpha$ -helix and a  $\beta$ -sheet contribute more to thermal stability than do the two disulfide bonds between (Cys40–Cys95) or within (Cys65–Cys72) a surface loop.<sup>103</sup>

Disulfide bonds, as covalent but sometimes transitory cross-links,<sup>105</sup> can be useful probes for elaborating protein folding pathways. With RNase A as with other proteins, folding has been studied by allowing the reduced protein to be oxidized by small-molecule disulfides such as oxidized glutathione (or oxidized dithiothreitol<sup>106</sup>), quenching the incomplete reaction by acidification or alkylation, and identifying the disulfide bonds in the folding intermediates. Both the acquisition and interpretation of such data on RNase A have been controversial. (For a review, see ref 102.) The controversy is due to the complexity of forming the four native disulfide bonds from eight cysteine residues. This complexity arises because eight cysteine residues can form 28 ( $= {}_8C_2$ ) distinct disulfide bonds. Moreover, a protein with eight cysteine residues can form 105 ( $= {}_8C_8 \times 7 \times 5 \times 3$ ) distinct species containing four disulfide bonds and 764 ( $= {}_8C_8 \times 7 \times 5 \times 3 + {}_8C_6 \times 5 \times 3 + {}_8C_4 \times 3 + {}_8C_2 + {}_8C_0$ ) distinct oxidized and reduced species, altogether. Indeed, RNase A with intentionally scrambled disulfide bonds has become a conventional substrate for enzymes, such as protein disulfide

isomerase, that catalyze the unscrambling of nonnative disulfide bonds.<sup>107,108</sup>

A more readily tractable issue in the folding of reduced RNase A involves the particular disulfide bond between Cys65 and Cys72. Along the polypeptide chain, Cys58 and Cys72 are equidistant from Cys65. According to polymer theory alone,<sup>105,109</sup> the stability of a disulfide bond between Cys58 and Cys65 should be equal to that of a disulfide bond between Cys65 and Cys72. Yet, in the M-peptide (which encompasses residues 50–79<sup>110</sup>), the Cys65–Cys72 disulfide bond is 3.6-fold more stable than is the Cys58–Cys65 disulfide bond.<sup>111</sup> This bias is consistent with the loop structure formed by the Cys65–Cys72 disulfide being a nucleation site for the folding of RNase A under oxidizing conditions.<sup>104,111–114</sup> Under reducing conditions, however, the Cys65–Cys72 disulfide bond is vulnerable. That bond and the Cys40–Cys95 disulfide bond are the first in native RNase A to suffer reduction by dithiothreitol.<sup>115</sup>

## B. Prolyl Peptide Bond Isomerization

RNase A exhibits a slow kinetic phase in its refolding (that is, its folding with native disulfide bonds intact).<sup>116</sup> The existence of this second kinetic phase is due to the presence of at least two distinct forms of unfolded RNase A.<sup>117–120</sup> If the native enzyme is unfolded rapidly and then allowed to refold immediately, all of the molecules refold rapidly. But if refolding is delayed, ~80% of the molecules refold slowly. The simplest kinetic scheme that is consistent with these data is



where N is the native enzyme,  $U_f$  are fast refolding species, and  $U_s$  are slow refolding species. The trans isomer of a typical peptide bond is greatly favored over the cis isomer. In contrast, a trans bond preceding a proline residue is only slightly favored, and its conversion to cis can be slow on the time scale of protein folding. In native RNase A, the peptide bonds to Pro42 and Pro117 are trans and those to Pro93 and Pro114 are cis. The isomerization of one or both of the cis peptide bonds may be responsible for the slow kinetic phase observed during the refolding of RNase A. The conservation of Pro93 and Pro114 in pancreatic ribonucleases from different vertebrates,<sup>20,22</sup> which is particularly rare for residues in a surface loop,<sup>121</sup> corroborates the importance of a cis peptide bond at these positions.<sup>122</sup>

The role of prolyl peptide bond isomerization in the refolding of RNase A has been probed by site-directed mutagenesis. The refolding rate of P42A RNase A is similar to that of the wild-type enzyme, indicating that cis–trans isomerization of the Pro42 peptide bond does not hinder refolding.<sup>123,124</sup> Nonetheless, a hydrogen bond from the side chain of Tyr97, which has the least mobile side chain of the six tyrosine and three phenylalanine residues,<sup>67</sup> to the Pro42 peptide bond enhances stability substantially.<sup>125,126</sup> The refolding kinetics of P93A, P114A, and P117A RNase A differ significantly from that of the wild-type enzyme.<sup>124</sup> This difference has allowed for an elaboration of the scheme in eq 2 to include additional species.<sup>124</sup>

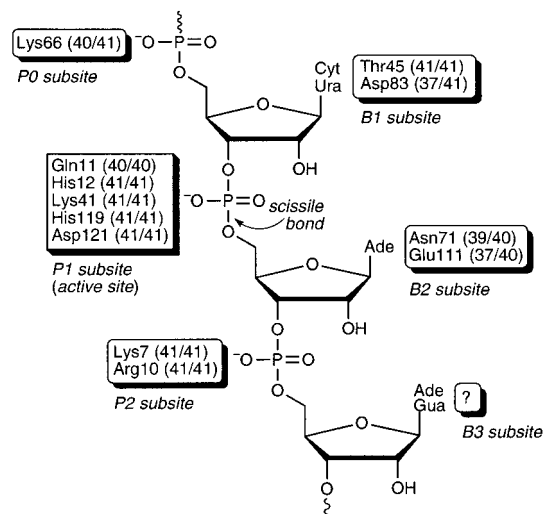
A major conclusion from work on the refolding of RNase A is that the Pro93 peptide bond is trans in the slowest refolding species.<sup>124,127,128</sup> In other words, the trans-to-cis isomerization of that bond is the slowest step in the refolding of the fully denatured enzyme. The kinetics of refolding suggest that the analogous peptide bond is cis in P93A RNase A.<sup>124,129</sup> Yet in the three-dimensional structure of crystalline P93G RNase A, this bond is trans because Gly93 allows the formation of a type II  $\beta$ -turn.<sup>130</sup>

## V. RNA Binding

The forces that lead to the binding of proteins to double-stranded DNA are becoming apparent.<sup>131–134</sup> By comparison, the forces that lead to the affinity and specificity of proteins for single-stranded RNA are relatively unknown.<sup>135</sup> RNase A is being used to reveal detailed information on the binding of proteins to RNA.

### A. Subsites

The number of lysine (10) and arginine (4) residues in RNase A exceeds that of aspartate (5) and glutamate (5) residues. Accordingly, RNase A is cationic ( $pI = 9.3$ <sup>136</sup>) at physiological pH. RNase A has been shown to destabilize double-stranded DNA by binding to single strands.<sup>137</sup> Moreover, cation titration<sup>138</sup> suggests that RNase A can occlude eleven nucleotides of a single-stranded nucleic acid<sup>139</sup> and that binding involves seven Coulombic interactions.<sup>140</sup> These results suggest that the interaction between the enzyme and a single-stranded nucleic acid extends well beyond the scissile bond. Structural<sup>74–78</sup> and functional (vide infra) data divulge the existence of several enzymic subsites (Figure 2). The subsites of RNase A have been the subject of recent reviews.<sup>141,142</sup>



**Figure 2.** Apparent interactions between the subsites in RNase A and a bound molecule of RNA. The 12 indicated residues have been shown by site-directed mutagenesis to make a contribution to substrate binding or turnover (or both). These residues are Lys7,<sup>158,159</sup> Arg10,<sup>158,159</sup> Gln11,<sup>37</sup> His12,<sup>154</sup> Lys41,<sup>153,155–157</sup> Thr45,<sup>146–148</sup> Lys66,<sup>159</sup> Lys71,<sup>149</sup> Asp83,<sup>148</sup> Glu111,<sup>149</sup> His119,<sup>154,46</sup> and Asp121.<sup>153,270</sup> Phe120 is also likely to contribute to the P1 subsite (via its main chain) and the B1 subsite (via its side chain).<sup>164</sup> The numbers in parentheses refer to the conservation of the indicated residues in pancreatic ribonucleases.<sup>20,22</sup>

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,<sup>74,81</sup> and demonstrates an approximately 30-fold kinetic preference for cytosine-containing versus uracil-containing substrates. In contrast, the B2 and B3 subsites bind all bases, but B2 has a preference for an adenine base<sup>143</sup> and B3 has a preference for a purine base.<sup>144,145</sup> Site-directed mutagenesis has been used to identify the most important residues in the B1<sup>146–148</sup> and B2<sup>149</sup> subsites. The existence of the B3 subsite has been inferred from kinetic data<sup>144,145</sup> and chemical modification studies.<sup>150–152</sup> In the crystalline RNase A·d(ApTpApApG) complex, the adenine base in the B3 subsite stacks with the adenine base in the B2 subsite.<sup>77</sup> The B3 “subsite” could therefore result from  $\pi$ - $\pi$  stacking interactions that stabilize the enzyme–nucleic acid complex solely by preorganization or desolvation of the nucleic acid.

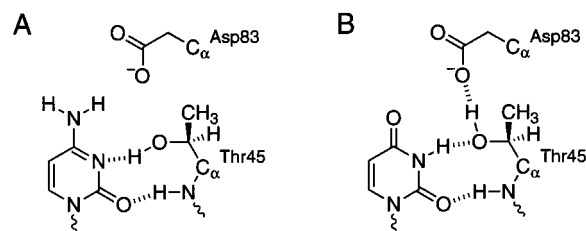
Three other enzymic subsites (P0, P1, and P2) interact with the phosphoryl groups of a bound substrate.<sup>141</sup> The enzyme catalyzes the cleavage of the P–O<sup>5'</sup> bond of a phosphoryl group bound in the P1 subsite, which is the active site (Figure 2). Site-directed mutagenesis has been used to identify the most important residues in the P1<sup>37,46,153–157</sup> and P2<sup>158,159</sup> subsites. The existence of the P0 subsite has been inferred from kinetic data,<sup>160,161</sup> molecular modeling,<sup>162</sup> and the results of recent site-directed mutagenesis experiments.<sup>159</sup>

## B. Substrate Specificity

RNase A catalyzes the cleavage of the P–O<sup>5'</sup> bond of an RNA strand and the hydrolysis of the P–O<sup>2'</sup> bond of a nucleoside 2',3'-cyclic phosphodiester (N>p) on the 3'-side of a pyrimidine residue. CpX is cleaved and C>p is hydrolyzed 2-fold faster than are the corresponding uridylyl substrates. (For a review, see ref 16.) Poly(C) is cleaved approximately 20-fold faster than is poly(U).<sup>146,163</sup> RNase A will also catalyze the cleavage of poly(A), but at a rate that is 10<sup>3</sup>- to 10<sup>4</sup>-fold less than that for the cleavage of poly(U).<sup>146,163</sup>

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.<sup>146</sup> In the structure of RNase A with uridine 2',3'-cyclic vanadate (U>v; see section VII), the O<sub>γ1</sub>–N<sub>3</sub> distance is 2.7 Å with a O<sub>γ1</sub>–H–N<sub>3</sub> angle of 147°, and the N–O<sub>2</sub> distance is 2.6 Å with a N–H–O<sub>2</sub> angle of 147°.<sup>164</sup> The side chain of Phe120 makes van der Waals contact with a pyrimidine base bound in the B1 subsite. The side chain of Ser123 has been assumed to form a hydrogen bond to a uracil bound in the B1 subsite, and to thereby enhance the rate of cleavage after uridine residues.<sup>165,166</sup> Such a hydrogen bond, however, is not evident in the RNase A complex with U>v.<sup>164,167</sup> Moreover, replacing the analogous serine in angiogenin, a homologue of RNase A, has no effect on substrate specificity.<sup>168</sup>

Site-directed mutagenesis has been used to create variants that cleave efficiently after a purine residue. Enzyme libraries were created in which all 20 amino acid residues replaced Thr45 or Phe120.<sup>147</sup> Screening these libraries revealed that replacing Thr45 with a



**Figure 3.** (A) Hydrogen bonds formed between a bound cytidine nucleotide and the residues of the B1 subsite of RNase A<sup>79</sup> and (B) hydrogen bonds formed between a bound uridine nucleotide and the residues of the B1 subsite of RNase A.<sup>164</sup>

glycine or alanine residue enables RNase A to cleave poly(A) efficiently.<sup>146</sup> The T45G and T45A enzymes have 10<sup>5</sup>- and 10<sup>3</sup>-fold increases, respectively, in poly-(A):poly(C) specificity with little compromise to catalytic efficacy. With its diminished substrate specificity, T45G RNase A is more effective than is the wild-type enzyme at degrading heteropolymeric RNA to completion,<sup>169</sup> which could be advantageous in ribonuclease protection assays.<sup>170</sup>

The interaction between Asp83 and Thr45 also affects the specificity of RNase A. Thermodynamic cycles with the T45G, D83A, and T45G/D83A variants indicate that the side chain of Asp83 has no effect on the kinetics of cleavage after cytidine residues, but does affect significantly the rate of cleavage of poly(U) and hydrolysis of U>p through an interaction that is dependent on the side chain of Thr45 (Figure 3).<sup>148</sup> Apparently, the Thr45–Asp83 hydrogen bond increases the ability of RNase A to cleave uridine-containing substrates by the selective stabilization of the transition state for this reaction. These results indicate that like a direct interaction between an enzyme and its substrate, an interaction between two functional groups within an enzyme can contribute to substrate specificity.

No alteration of Phe120 produced an enzyme that catalyzes the efficient cleavage of RNA after purine residues.<sup>146</sup> This result is consistent with two structural features of Phe120 that are apparent in the RNase A·U>v complex.<sup>164,171</sup> First, the aromatic ring of Phe120 appears to interact with a pyrimidine base bound in the B1 subsite. The structural difference between a pyrimidine base and a purine base is largely two-dimensional, in the plane of the  $\pi$ -system. Hence, the side chain of Phe120 does not mediate purine:pyrimidine specificity, but acts as a hydrophobic mattress on which a base lies. Second, the main-chain nitrogen of Phe120 forms a hydrogen bond with a nonbridging oxygen atom of the reacting phosphoryl group. (See section VIII.) Thus, even if the side chain of Phe120 did mediate substrate specificity, changing this residue could hamper catalysis.

## C. One-Dimensional Diffusion

Diffusion is a barrier on the free energy landscape of every bimolecular process.<sup>172</sup> The ability to diffuse in one dimension can accelerate the formation of a site-specific interaction within a linear biopolymer by up to 10<sup>3</sup>-fold.<sup>173</sup> Such facilitated diffusion is used by transcription factors and restriction endonucleases

to locate specific sites on double-stranded DNA.<sup>174,175</sup> The rapid cleavage of single-stranded DNA by BAL 31 nuclease has been interpreted as arising from facilitated diffusion.<sup>176</sup> The backbone of RNA, like that of DNA, could allow for the one-dimensional diffusion of proteins.<sup>177</sup>

The facilitated diffusion of a protein along RNA has been demonstrated with RNase A.<sup>178</sup> Evidence for facilitated diffusion has been obtained using an RNA/DNA chimera. Specifically, a uridine nucleotide is cleaved more quickly by RNase A if it is flanked by a long stretch of poly(dA) than if it is flanked by a short stretch. This advantage is lost if the salt concentration is high, as expected from a Coulombic interaction between the cationic enzyme and an anionic nucleic acid. Facilitated diffusion may enable cytotoxic homologues of RNase A (see section XII) 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.

#### D. Processive Catalysis

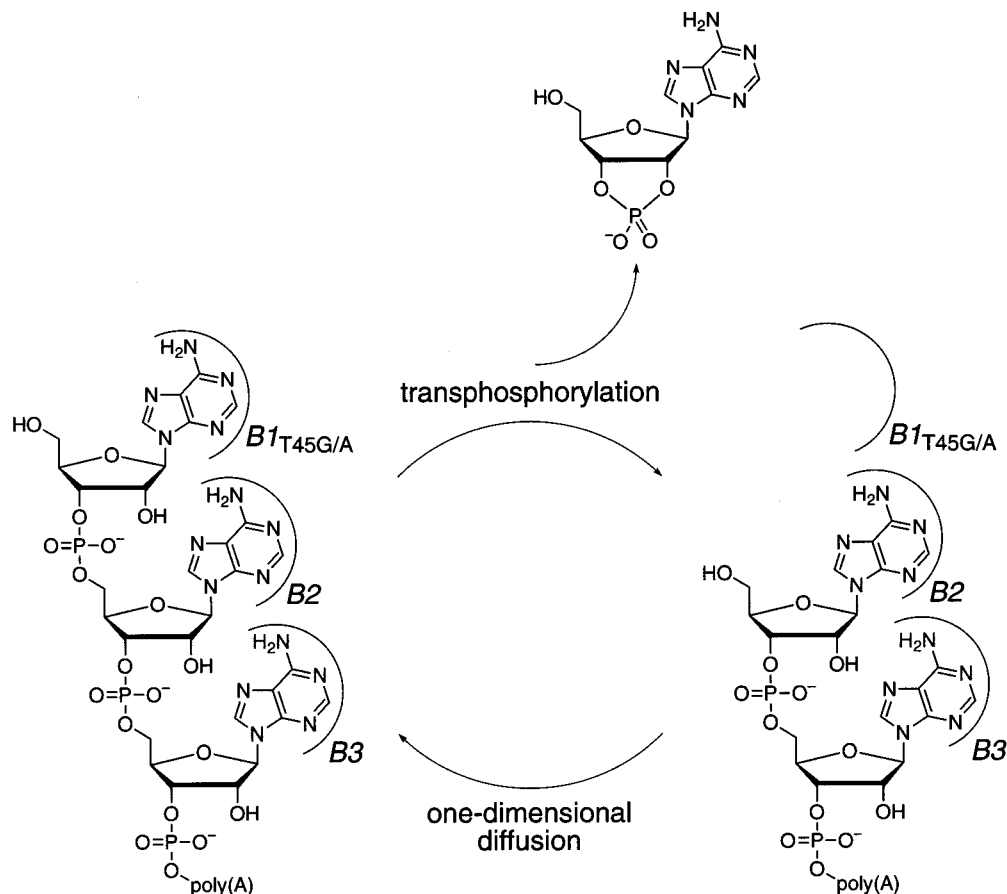
"Distributive" enzymes bind a polymeric substrate, catalyze a chemical reaction, and release to solvent a polymeric product. In contrast, "processive" enzymes bind a polymeric substrate and catalyze a series of identical chemical reactions along that polymer before releasing it to solvent. Many enzymes that catalyze the synthesis and degradation of nucleic acids do so processively.<sup>179</sup> The cleavage of poly(C) and poly(U) by wild-type RNase A and the T45G and

T45A variants are distributive, as revealed by <sup>31</sup>P NMR and order-of-addition experiments. In contrast, the cleavage of poly(A) by both variants is processive.<sup>146,147</sup>

For a substrate to be acted on processively, it must contain a repeating structural motif. Poly(C), poly(U), and poly(A) have repeating motifs, such as a ribosyl group, phosphoryl group, and base. Yet, none of these polymers is cleaved processively by wild-type RNase A. The distributive behavior of RNase A is likely to arise from the opposing specificities of the B1 subsite (which does not bind adenine<sup>74,81</sup>) and the B2 and B3 subsites (which bind cytosine and uracil only weakly<sup>143-145</sup>). 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 4). 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.<sup>146</sup>

#### VI. Substrates

Early work on the kinetics of catalysis by RNase A used substrates that were either ill-defined heterogeneous strands of RNA (for example, "yeast RNA"<sup>180</sup>) or nucleoside 2',3'-cyclic phosphodiester,<sup>181</sup> which are the products rather than the substrates of the germinal transphosphorylation reaction (see

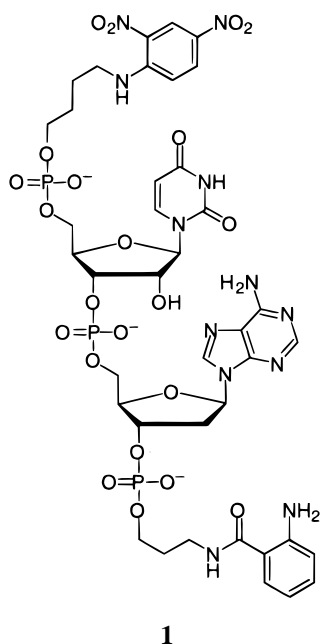


**Figure 4.** Putative mechanism for the processive cleavage of poly(A) by T45G RNase A and T45A RNase A.<sup>146</sup> The enlarged B1 subsite in these variants can accommodate an adenine base.

section IX).<sup>182,183</sup> One appropriate application of assays using RNA polymers is for the detection of ribonucleolytic activity in a complex mixture. For example, the release of methylene blue from yeast RNA provides a sensitive assay at 688 nm, a wavelength of light not absorbed by most biomolecules.<sup>184</sup> Alternatively, zymogram assays can detect as little as 1 pg (0.1 fmol) of RNase A. In a zymogram assay, a polymeric substrate is incorporated into a gel, and cleavage is visualized by staining for intact polymers after electrophoresis<sup>185–187</sup> or isoelectric focusing.<sup>188</sup> A zymogram blot is also effective.<sup>189</sup>

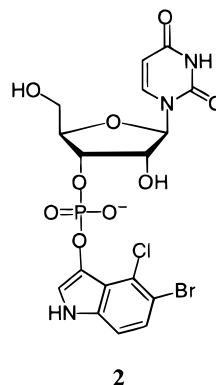
Answering questions about enzymatic catalysis with chemical rigor requires the use of well-defined substrates. Homopolymeric substrates such as poly(U) and poly(C) are now readily available. Further, the advent of phosphoramidite chemistry has enabled the facile synthesis of any di-, tri-, or tetranucleotide substrate. (For an example, see ref 190.) Uridyl-(3'→5')adenosine (UpA) and cytidyl-(3'→5')adenosine (CpA), which have well-defined extinction coefficients,<sup>191</sup> have become the most often used oligonucleotide substrates. Because RNase A does not catalyze DNA cleavage, the synthesis of RNA/DNA chimeras extends further the horizon of possible analyses.<sup>178,192</sup>

A new fluorogenic substrate provides the basis for an extremely sensitive assay for RNase A. 5'-[O-[4-[(2,4-Dinitrophenyl)amino]butyl]phosphoryl]uridylyl-(3'→5')2'-deoxyadenosine 3'-[N-[(2-aminobenzoyl)amino]prop-3-yl] phosphate (DUPAAA; **1**) consists of a fluorophore (*o*-aminobenzoic acid) linked via Ud(pA) to a quencher (2,4-dinitroaniline).<sup>193</sup> Cleavage of the phosphodiester bond in the Ud(pA) linker results in a 60-fold increase in fluorescence, enabling the detection of a 50 fM concentration of RNase A.



New chromogenic substrates facilitate assays of RNase A. Uridine 3'-(5-bromo-4-chloroindol-3-yl) phosphate (U-3'-BCIP; **2**) is a substrate for RNase A.<sup>194,195</sup> The 5-bromo-4-chloroindol-3-ol product dimerizes rapidly in air to form a blue pigment. This

substrate is analogous to (5-bromo-4-chloroindol-3-yl)galactose (X-gal), a common substrate for  $\beta$ -galactosidase. Other chromogenic substrates rely on the production of yellow phenolates from the cleavage of uridine 3'-aryl phosphates.<sup>37,154,196</sup>



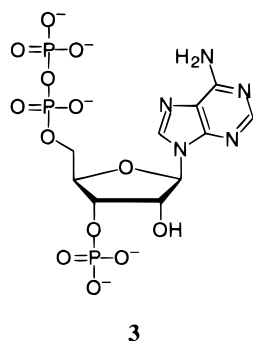
## VII. Inhibitors

The most potent inhibitor of RNase A, appropriately called "ribonuclease inhibitor" (RI), is a 50-kDa protein that constitutes  $\leq 0.01\%$  of the protein in the cytosol of mammalian cells.<sup>18,197</sup> RI presumably protects cytosolic RNA against the invasion of pancreatic ribonucleases. The value of  $K_d$  for the RI·RNase A complex has been measured to be  $4.4 \times 10^{-14}$  M<sup>198</sup> and  $6.7 \times 10^{-14}$  M.<sup>199</sup> The crystalline structures of RI<sup>200</sup> and the RI·RNase A complex<sup>201,202</sup> disclose that this tight association is due largely to hydrogen bonds and Coulombic interactions. The ability to evade RI appears to be a key attribute of those homologues of RNase A that are cytotoxic. (See section XII.) RI has been the object of recent reviews.<sup>203,204</sup>

Small-molecule inhibitors of RNase A are also known. Nucleosides form complexes with oxovanadium(IV) and vanadium(V) ions. At least one of these complexes with vanadium(V), uridine 2',3'-cyclic vanadate (U>v), is a potent inhibitor of RNase A. Uridine–vanadate complexes have been reported to inhibit RNase A with an apparent  $K_i$  near  $10 \mu\text{M}$ .<sup>205</sup> In a detailed study, the value of  $K_i$  for the U>v species alone has been determined to be near  $0.5 \mu\text{M}$ .<sup>206</sup>

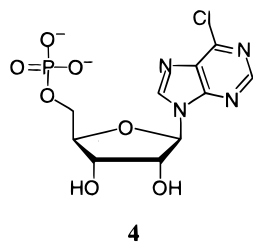
U>v was conceived as a transition-state analogue for the hydrolysis reaction of RNase A.<sup>207</sup> The vanadium in U>v does indeed have a nearly trigonal bipyramidal geometry when bound in the active site of RNase A.<sup>164,167</sup> Nevertheless, both theoretical<sup>208</sup> and experimental<sup>157</sup> approaches reveal that U>v more closely resembles the ground-state rather than the transition state of the RNase A·U>p complex.

The most potent noncovalent small-molecule inhibitors of RNase A are now 5'-diphosphoadenosine 3'-phosphate (**3**) and 5'-diphosphoadenosine 2'-phosphate.<sup>209</sup> The value of  $K_d$  for the RNase A·**3** complex is  $0.24 \mu\text{M}$ , and that for the RNase A·5'-diphosphoadenosine 2'-phosphate complex is  $0.52 \mu\text{M}$ . The structures of crystalline complexes reveal that the bound inhibitors occupy the P1 and B2 subsites.<sup>210</sup>

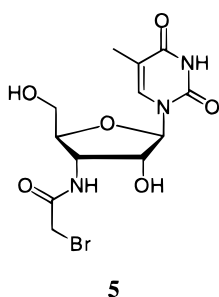


Nucleophilic aromatic substitution by poly(A) on 1-fluoro-2,4-dinitrobenzene yields poly[2'-*O*-(2,4-dinitrophenyl)]poly(adenylic acid) [(DNP-poly(A)].<sup>211</sup> DNP-poly(A) of molecular mass 110 kDa and DNP:adenine ratio of 1:1.5 is a reversible competitive inhibitor but not a substrate of RNase A, RNase B, RNase S (see section X), and other ribonucleases.<sup>212</sup> The  $IC_{50}$  values for inhibition of RNase A, RNase B, and RNase S by DNP-poly(A) have been reported to be 3.20, 0.50, and 0.08  $\mu$ M, respectively.<sup>212</sup> Encapsulating DNP-poly(A) within porous gels<sup>213</sup> or attaching it to acrylic beads<sup>212</sup> generates affinity matrixes that effectively remove RNase A from solution. Bound RNase A can be eluted from these matrixes by washing with aqueous solutions of high ionic strength.

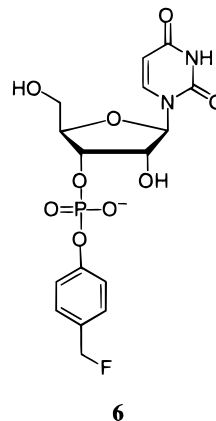
Specific affinity labels for RNase A exist. 6-Chloropurine 9- $\beta$ -D-ribofuranosyl 5'-monophosphate (**4**) alkylates the  $\alpha$ -amino group of Lys1, presumably after binding to the B3 subsite (Figure 2).<sup>150,151</sup> The



structure of the crystalline product of the alkylation of RNase A by **4** is known.<sup>152</sup> 2'-(3'-*O*-Bromoacetyluridine<sup>214,215</sup> and its amide analogues 3'-(bromoacetamido)-3'-deoxythymidine (**5**), 3'-(bromoacetamido)-3'-deoxyuridine, 3'-(bromoacetamido)-3'-deoxyarabino-furanosyluracil, 2'-(bromoacetamido)-2'-deoxyuridine, and 2'-(bromoacetamido)-2'-deoxyxylofuranosyl-uracil<sup>216-218</sup> alkylate the side chains of His12 or His119. The structures of the crystalline products of the alkylation of RNase A by **5** and by 3'-(bromoacetamido)-3'-deoxyuridine are known.<sup>219</sup>

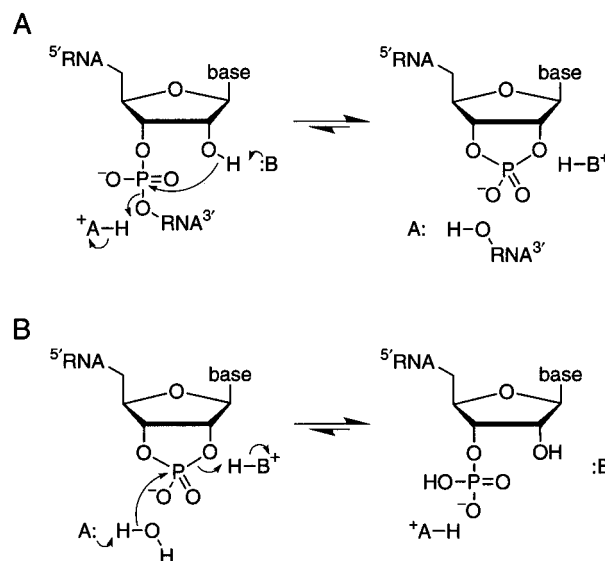


Finally, RNase A has been the object of mechanism-based inactivation. The enzyme catalyzes the conversion of uridine 3'-[4-(fluoromethyl)phenyl] phosphate (**6**) to a quinone methide, which likely alkylates the side chain of Lys7, Arg10, Gln69, or Glu111 (Figure 2).<sup>220</sup> None of these residues are in the active site, and approximately one-third of the catalytic activity remains after alkylation.



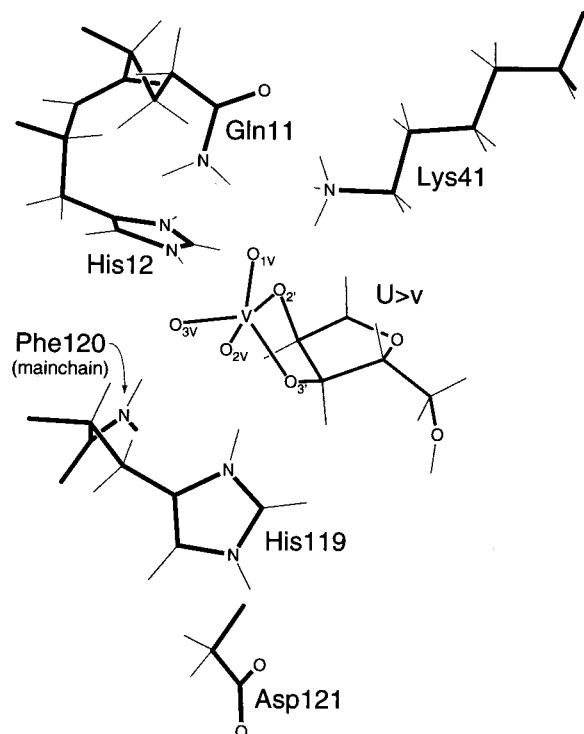
### VIII. Reaction Mechanism

RNase A catalyzes the cleavage of the P-O<sup>5'</sup> bond of RNA. Figure 5 depicts a mechanism of catalysis that is consistent with all known data from work on the enzyme itself.<sup>221</sup> Other mechanisms have also been proposed (vide infra).<sup>222-225</sup> In the mechanism in Figure 5, 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.<sup>226,227</sup> 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 nucleoside 2',3'-cyclic phosphodiester occurs in a separate process (see section IX), and resembles the



**Figure 5.** (A) Putative mechanism for the transphosphorylation reaction catalyzed by RNase A and (B) putative mechanism for the hydrolysis reaction catalyzed by RNase A.<sup>221</sup> In both mechanisms, "B" is His12 and "A" is His119.





**Figure 6.** Crystalline structure of the active site of RNase A bound to uridine 2',3'-cyclic vanadate (U>v). The structure was refined at 2.0 Å from X-ray and neutron diffraction data collected from crystals grown at pH 5.3.<sup>164</sup> The side chain of Phe120 and the uracil base are not shown.

reverse of transphosphorylation. Both reactions shown in Figure 5 probably occur via transition states having a pentavalent phosphorus atom. The side chain of Lys41 and the main chain of Phe120 enhance catalysis by stabilizing this transition state (vide infra).

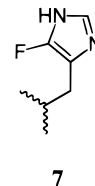
The high-resolution structure of the crystalline complex of RNase A and U>v obtained by joint X-ray/neutron diffraction analysis has provided invaluable insight into the catalytic mechanism of RNase A.<sup>164</sup> This resolution has been extended to 1.3 Å.<sup>167</sup> The active site of this structure is shown in Figure 6. In the active site, the side chains of His12, His119, Lys41, and Gln11, and the mainchain of Phe120 are all proximal to the vanadyl group. The apparent roles of these side chains (and that of Asp121) in catalysis are described below. The main-chain nitrogen of Phe120 donates a hydrogen bond to a nonbridging oxygen, O<sub>3v</sub> (N–O<sub>3v</sub> distance = 2.9 Å, N–H–O<sub>3v</sub> angle = 162°). No data exist on the role of the main-chain nitrogen of Phe120 in catalysis.

### A. His12 and His119

Histidines were identified as important residues in early work on RNase A. Specifically, haloacetates were shown to carboxymethylate the histidine residues of RNase A.<sup>15,228–231</sup> When the proper conditions are effected, only one histidine residue is alkylated in each molecule of RNase A. The rate of the single enzymic carboxymethylation is nearly 10<sup>4</sup>-fold greater than that of free histidine (and greater than that of enzymic carbamoylmethylation), which is consistent with the binding of the anionic haloacetate in the

cationic active site. The alkylation, which causes a marked decrease in catalytic activity, modifies only His12 or His119.

Catalysis by RNase A has a classic bell-shaped pH-rate profile.<sup>222,232,233</sup> This profile is consistent with a mechanism that involves two titratable residues, one protonated and the other unprotonated. His12 and His119 are the only residues that need be invoked to explain the pH dependence of catalysis. Recent support for this assignment comes from the semi-synthesis of an RNase A variant containing a 4-fluorohistidine residue (7) at both position 12 and position 119 of RNase A.<sup>234</sup> The pH dependence of this



variant is still bell-shaped, but shifted to lower pH. Because 4-fluorohistidine has a lower pK<sub>a</sub> than does histidine, this perturbation is consistent with both 4-fluorohistidine residues participating in catalysis. These data contradict the conclusion of an earlier study in which substituting 4-fluorohistidine at position 12 of RNase S (see section X) was reported to yield an inactive enzyme that was isostructural with native RNase S.<sup>235</sup>

Recombinant DNA techniques have been used to produce RNase A variants in which either His12 or His119 is replaced with an alanine residue.<sup>154</sup> The second-order rate constant,  $k_{cat}/K_m$ , is proportional to the association constant of an enzyme and the rate-limiting transition state during catalysis.<sup>236</sup> Eliminating the imidazole group of His12 decreases the affinity of the enzyme for this transition state by 10<sup>4</sup>-fold during cleavage of poly(C), UpA, and UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>. Eliminating the imidazole group of His119 decreased this affinity by 10<sup>4</sup>-fold during cleavage of poly(C) and by almost 10<sup>4</sup>-fold during cleavage of UpA. In contrast, this change had no significant effect on the rate of 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<sub>a</sub> of the conjugate acid of the leaving groups. The nucleoside leaving groups in poly(C) and UpA have conjugate acids with pK<sub>a</sub> ≈ 14.8 (which is the pK<sub>a</sub> of CH<sub>3</sub>OCH<sub>2</sub>CH<sub>2</sub>OH<sup>237</sup>). 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<sub>a</sub> = 7.14.<sup>238</sup> Thus, the contribution of His119 to catalysis decreases when the pK<sub>a</sub> of the conjugate acid of the leaving group decreases. This finding is the strongest evidence to date that the role of His119 is to protonate the leaving group during RNA cleavage. In addition, Brønsted analyses of catalysis by wild-type RNase A ( $\beta_{lg} = -0.19$ <sup>196</sup>) and imidazole ( $\beta_{lg} = -0.59$ <sup>239</sup>) are consistent with general acid catalysis in the enzymic reaction.

No analogous evidence for the mechanistic role of His12 is available from kinetic data. One attempt has been made to attain such evidence. If His12 does indeed act as a base, then His12 is likely to contribute less to the enzymic 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  $pK_a = 8.2$  by kinetic and thermodynamic measurements,<sup>240</sup> but the 2'-hydroxyl group has  $pK_a = 12.5$  by kinetic measurements<sup>241</sup> and  $pK_a = 13.9$  by thermodynamic measurements.<sup>242</sup> Yet, 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>.<sup>243</sup> Likewise, 2'-deoxy-2'-thio-UpU appears not to be a substrate.<sup>244</sup> Among 2'-oxo nucleotides, UpA is cleaved faster by RNase A than is UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> or UpU.<sup>16,154</sup> Accordingly, 2'-deoxy-2'-thio-UpA was synthesized and its interaction with RNase A was studied in detail.<sup>243</sup> 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 the cleavage of this 2'-thiol nucleotide analogue are at least 10<sup>5</sup>-fold lower than are those for the cleavage of UpA.<sup>243</sup> The basis for such poor catalysis is unclear. Nonetheless, because His119 has been identified as the acid for the cleavage reaction, it seems reasonable to put forth His12 as the base.

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

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

and

$$\frac{k_{\text{wild-type}}}{k_{\text{H119A}}} = \left( \frac{K_a^{\text{His119}}}{K_a^{\text{H}_2\text{O}}} \right)^\alpha$$

where  $pK_a^{\text{His12}} = 5.8$  and  $pK_a^{\text{His119}} = 6.2$ ,<sup>245</sup> and  $pK_a^{\text{H}_3\text{O}^+} = -1.7$  and  $pK_a^{\text{H}_2\text{O}} = 15.7$ . The Brønsted equation therefore predicts that general base catalysis provides a 10<sup>7.5β</sup>-fold rate enhancement, and general acid catalysis provides a 10<sup>9.5α</sup>-fold rate enhancement. Values of  $\alpha$  and  $\beta$  tend to be approximately 0.5 for proton transfers between oxygen and nitrogen.<sup>246</sup> Thus, the rate enhancements predicted with this simple model are similar to those observed by experiment.

His119 has also been replaced by an asparagine residue.<sup>46</sup> This substitution decreases the affinity of the enzyme for the rate-limiting transition state by 10<sup>2</sup>-fold during the cleavage of poly(C) and UpA. An asparagine residue, unlike an alanine residue, can donate a hydrogen bond to the leaving group in the transition state. One interpretation of these data is that such a hydrogen bond can enhance the affinity of the enzyme for the transition state by 10<sup>2</sup>-fold.

Finally, the results of experiments in imidazole buffer (but in the absence of enzyme) have been used to argue for a different role for His119 in catalysis by RNase A. Specifically, RNase A has been proposed to catalyze RNA cleavage via a triester mechanism.<sup>225</sup> In this mechanism, His119 is proposed to both protonate a nonbridging oxygen of the phos-

phate anion and deprotonate this same oxygen in a phosphorane intermediate. The evidence for and against a triester mechanism in the buffer-catalyzed cleavage of RNA has been a subject of recent reviews.<sup>247,248</sup> Some textbooks (cf. refs 249 and 250) present the triester mechanism as the one operating in the enzymic active site. The results of at least three experiments on the enzyme itself provide direct evidence against this view. First, wild-type RNase A and the H119A variant cleave UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> at the same rate.<sup>154</sup> These data preclude the participation of His119 in the formation or breakdown of a phosphorane, at least during the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub>.<sup>251</sup> Second, catalysis by RNase A has small thio effects, which are rate effects upon substitution of a nonbridging phosphoryl oxygen with sulfur.<sup>252,253</sup> These data have been used to argue against the triester mechanism,<sup>254</sup> although correlation of the thio effects with the chirality of the enzymic transition state and considerations of the identity of the rate-limiting transition state somewhat weaken this argument.<sup>247,251</sup> Third, kinetic isotope effect data on the cleavage of <sup>18</sup>O-labeled UpOCH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>-*m*-NO<sub>2</sub> by RNase A are inconsistent with a triester mechanism. Rather, these data support a concerted mechanism in which the transition state is slightly associative.<sup>255</sup>

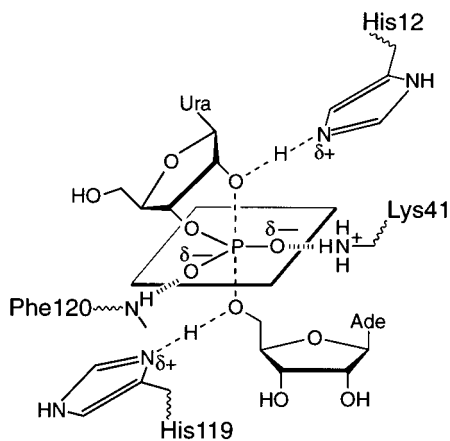
Why does RNase A not use the triester mechanism? In the active site of RNase A, the desolvated side chains of His12 and His119 are aligned to interact simultaneously as a base and acid with a bound, desolvated substrate (Figure 6). Such an alignment of two imidazolyl groups is implausible in imidazole buffer and improbable in an enzyme mimic. Thus, the enzyme can access a reaction coordinate that is relatively unavailable in nonenzymic systems.

## B. Lys41

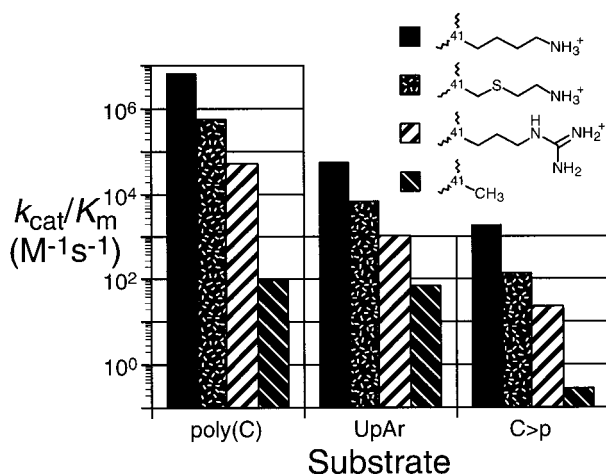
Early chemical modification work suggested that Lys41 contributes to catalytic activity.<sup>256</sup> This finding was confirmed when a variant in which Lys41 is replaced by an arginine residue was shown to have approximately 2% of the activity of the wild-type enzyme for C>p hydrolysis.<sup>153</sup> These studies demonstrated the importance, but not the role, of Lys41 in catalysis.

The catalytic role most commonly attributed to Lys41 is to stabilize the excess negative charge that accumulates on the nonbridging phosphoryl oxygens in the transition state during RNA cleavage (Figure 7). It has been assumed that this stabilization occurs by Coulombic interactions.<sup>153,236,257,258</sup> But, it has also been proposed that the stabilization occurs by way of a short, strong hydrogen bond involving the partial transfer of a proton from Lys41.<sup>259</sup>

To probe the role of Lys41 in catalysis, cysteine elaboration was used to introduce nonnatural amino acid residues at position 41.<sup>155</sup> Specifically, Lys41 was replaced by a cysteine residue, which was then alkylated with five different haloalkylamines. In the resulting enzymes, high values of  $k_{cat}/K_m$  for poly(C) cleavage correlate with low values of side chain  $pK_a$ . The presence of an amidino side chain, which can donate a second hydrogen bond, does not enhance activity. An enzyme with a quaternary amino group



**Figure 7.** Putative structure of the transition state during transphosphorylation of UpA by RNase A. The dissociation constant for this complex is  $K_{TX} \leq 2 \times 10^{-15}$  M.<sup>156</sup>



**Figure 8.** Values of  $k_{cat}/K_m$  for catalysis of the transphosphorylation of poly(C) and UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub> (UpAr) and the hydrolysis of C>p by wild-type RNase A, K41[S-(aminoethyl)cysteine] RNase A, K41R RNase A, and K41A RNase A. Assays were performed at 25 °C in 0.10 M sodium 2-(*N*-morpholino)ethanesulfonate buffer, pH 6.0, containing NaCl (0.10 M).<sup>157</sup>

in the side chain of residue 41 has low activity. These data support a model in which the role of Lys41 is not merely Coulombic, but is to donate a single hydrogen bond to the transition state during catalysis.

The role of Lys41 appears to be similar in catalysis of both the transphosphorylation and the hydrolysis reactions (Figure 5). Wild-type RNase A and variants in which Lys41 is replaced by alanine, arginine, and *S*-(aminoethyl)cysteine were assayed for their abilities to catalyze transphosphorylation [of poly(C) and UpOC<sub>6</sub>H<sub>4</sub>-p-NO<sub>2</sub>] and hydrolysis (of U>p). The relative  $k_{cat}/K_m$  values are similar for the four enzymes, regardless of the substrate (Figure 8).<sup>157</sup> These data are consistent with Lys41 donating a single hydrogen bond in the transition state of both reactions catalyzed by RNase A. A comparison of catalysis by K41A RNase A and the wild-type enzyme shows that this hydrogen bond lowers the free energy of the rate-limiting transition state for poly(C) cleavage by 5 kcal/mol. In contrast to catalysis by the wild-type enzyme, a change in covalency limits catalysis by K41A RNase A.<sup>156</sup>

### C. Asp121

In native RNase A, Asp121 can interact with His119, the acid in the catalysis of RNA cleavage. The interaction between His119 and Asp121 defines a motif known as the catalytic dyad, in which a histidine residue that mediates general acid/base catalysis forms a hydrogen bond with an aspartate residue. This motif bears a striking resemblance to the conserved motif known as the catalytic triad found in serine proteases. (For reviews, see refs 260 and 261.)

Several attempts have been made to determine the role of aspartate in the catalytic dyad of RNase A. In one study, Asp121 was replaced with asparagine in a semisynthetic enzyme. This semisynthetic ribonuclease, RNase(1–118)·(111–124),<sup>262,263</sup> consists of a noncovalent complex between residues 1–118 of RNase A (obtained from proteolytic digestion of RNase A), and an overlapping synthetic peptide composed of the 14 C-terminal residues of RNase A, except with Asp121 replaced by an asparagine residue. The D121N semisynthetic variant has approximately 5% of the catalytic activity of the analogous wild-type semisynthetic enzyme.<sup>264</sup> These data are difficult to interpret, however, because the three-dimensional structure D121N semisynthetic variant deviates from that of RNase(1–118)·(111–124).<sup>265–267</sup>

Site-directed mutagenesis has been used to replace Asp121 with glutamate, asparagine, and alanine residues.<sup>36,153,268–270</sup> The glutamate variant has approximately 17% of the activity of the wild-type enzyme for C>p hydrolysis.<sup>153</sup> The crystalline structures of the other two variants were determined by X-ray diffraction analysis to a resolution of 1.6 Å with an *R* factor of 0.18.<sup>270</sup> The alterations do not perturb the conformation of the enzyme. In the structure of D121N RNase A, N<sub>δ</sub> rather than O<sub>δ</sub> of Asn121 faces His119. The values of  $k_{cat}/K_m$  and  $k_{cat}$  for transphosphorylation of UpA and poly(C) are reduced by 10<sup>1</sup>-fold (D121N) and 10<sup>2</sup>-fold (D121A).<sup>270</sup> The values of  $k_{cat}/K_m$  and  $k_{cat}$  for hydrolysis of U>p are reduced by 3-fold (D121N) and 10-fold (D121A). The alterations do not otherwise effect the pH-rate profiles for hydrolysis. These decreases are far less than those observed for analogous variants of serine proteases.<sup>271–275</sup> 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 than do the analogous hydrogen bonds in serine proteases.<sup>276,277</sup>

Replacing Asp121 with an asparagine or alanine residue results in a loss of conformational stability at pH 6.0 of  $\Delta\Delta G_m = -2.0$  kcal/mol, from a total  $\Delta G_m = 9.0$  kcal/mol.<sup>269</sup> This loss is similar in magnitude to the loss of transition-state binding during catalysis of RNA cleavage. Thus, a major role of the

His $\cdots$ Asp catalytic dyad is to enhance the conformational stability of the enzyme. The pH dependencies of the conformational stabilities of the wild-type, D121N, 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  $pK_a$ .

The kinetics of catalysis by D121N RNase A and D121A RNase A illuminate another aspect of the mechanism of RNase A. The side chain of His119 can occupy two conformations that differ by rotation about the  $C_\alpha-C_\beta$  bond. In one of these conformations (position A), the side chain of His119 forms a hydrogen bond with the side chain of Asp121. In the other conformation (position B), the side chain of His119 forms a hydrogen bond with solvent. In the three-dimensional structures of RNase A bound to d(CpA) and cytidyl(2'→5')adenosine, the adenine base prevents His119 from being in position B.<sup>79,278</sup> Thus, His119 must act from position A during catalysis of transphosphorylation. But structural data show that His119 could act from either position A or position B during catalysis of hydrolysis. Indeed, it has been suggested that the A $\rightleftharpoons$ B equilibrium evolved to enable transphosphorylation to occur with His119 in position A and hydrolysis to occur with His119 in position B.<sup>279</sup> Yet, RNase A with an aspartate, asparagine, or alanine residue in position 121 have differential abilities to catalyze hydrolysis.<sup>269</sup> This result suggests that residue 121 is proximal to His119 during catalysis of hydrolysis—hydrolysis can occur with His119 in position A.

#### D. Gln11

X-ray diffraction analyses show that the side chain of Gln11 can form a hydrogen bond to a substrate, substrate analogue, phosphate ion, or sulfate ion bound in the active site of RNase A. (For a review of these analyses, see ref 82.) <sup>1</sup>H NMR spectroscopy provides further evidence for this interaction, as large changes in the  $NH_{\epsilon 1}$  and  $NH_{\epsilon 2}$  resonances of Gln11 are observed upon binding of pyrimidine nucleotides.<sup>280</sup> In the high-resolution structure of RNase A complexed with U $\nu$  (Figure 6), the side-chain nitrogen of Gln11 forms a hydrogen bond with the nonbridging oxygen  $O_{1V}$  ( $N_{\delta 2}-O_{1V}$  distance = 2.6 Å,  $N_{\delta 2}-H-O_{1V}$  angle = 140°).<sup>164</sup> A study of semisynthetic variants of RNase S (see section X) having various residues at position 11 have also ascribed a significant role for Gln11 in catalysis.<sup>281</sup> Together, these data portend an important role for Gln11 in the catalytic mechanism of RNase A.

The role of Gln11 in catalysis by RNase A has been probed by creating variants in which this residue is replaced with alanine, glutamine, and histidine.<sup>37</sup> The results show that Gln11 does not stabilize the rate-limiting transition state during catalysis by RNase A. Rather, Gln11 serves to increase the free energy of the enzyme-substrate complex.

The destabilization of the enzyme-substrate complex may be an obligatory event in the evolution of enzymatic efficiency,<sup>236,282,283</sup> and can arise from a variety of molecular scenarios. In RNase A, the increase in the free energy of the Michaelis complex appears to be due (at least in part) to a binding

interaction that reduces nonproductive binding. In the absence of the side chain of Gln11, the active site 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  $k_{cat}$  and an identical decrease in the value of  $K_m$ , such that the value of  $k_{cat}/K_m$  is unchanged.<sup>236</sup> This effect is most dramatic in the turnover of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> by Q11A RNase A. This substrate, unlike poly(C) or UpA, cannot interact with enzymic subsites on both sides of the scissile bond, making its proper alignment problematic. The values of both  $k_{cat}$  and  $K_m$  for the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> by Q11A RNase A are 10<sup>2</sup>-fold lower than those for the cleavage of UpOC<sub>6</sub>H<sub>4</sub>-*p*-NO<sub>2</sub> 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.

### IX. Reaction Energetics

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 enzymes such as triosephosphate isomerase and proline isomerase,<sup>284</sup> which catalyze the relatively isogonic interconversion of a single substrate and a single product. Regardless, progress has been made with RNase A.

#### A. Transphosphorylation versus Hydrolysis

RNase A catalyzes both the transphosphorylation of RNA to form a 2',3'-cyclic phosphodiester intermediate and hydrolysis of this cyclic intermediate to form a 3'-phosphomonoester (Figure 5).<sup>285,286</sup> <sup>31</sup>P NMR spectroscopy<sup>287,288</sup> has been used to monitor in a continuous assay the extent to which the 2',3'-cyclic phosphodiester intermediate accumulates during catalysis by RNase A and small molecules.<sup>183</sup> <sup>31</sup>P NMR spectra show that the cyclic intermediate accumulates during catalysis by RNase A. The enzyme releases rather than hydrolyzes most of the 2',3'-cyclic phosphodiester product of RNA transphosphorylation, a result in accord with earlier chromatographic analyses.<sup>182,285,286</sup> In contrast, the cyclic intermediate does not accumulate during catalysis by hydroxide ion or imidazole buffer.<sup>183</sup> In the presence of these small-molecule catalysts, hydrolysis of the cyclic intermediate is faster than transphosphorylation of RNA.

A trapping experiment has been used to evaluate the "throughput" of the reaction catalyzed by RNase A. [5,6-<sup>3</sup>H]UpA was incubated with RNase A in the presence of excess unlabeled uridine 2',3'-cyclic phosphodiester, which dilutes the specific radioactivity of any released cyclic intermediate. Only 0.1% of the RNA substrate was found to be both transphosphorylated and hydrolyzed without dissociating from the

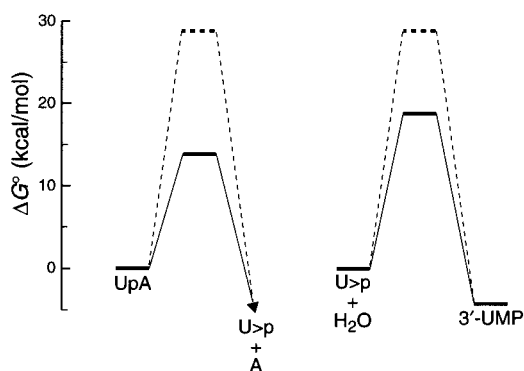
enzyme. These results suggest that RNase A has evolved primarily to catalyze transphosphorylation rather than hydrolysis. [To denote this preference, perhaps RNase A should be referred to (once again<sup>15</sup>) as an “RNA depolymerase”.] Many textbooks (cf., refs 250 and 289–292) incorrectly picture the mechanism of RNA hydrolysis by RNase A as proceeding in one two-step process rather than in two one-step processes (Figure 5).<sup>182,183</sup>

The result of the throughput experiment 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 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. But RNase A short-circuits this cycle by releasing rather than hydrolyzing the cyclic intermediate. Thus, RNase A has an iso mechanism<sup>293,294</sup> in which the protonation states of the unliganded enzyme are interconverted by a pathway that does not involve substrate molecules.

## B. Rate Enhancement

The products of the uncatalyzed cleavage of UpA are the same as those in the enzyme-catalyzed reaction.<sup>156</sup> 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  $k_{\text{cat}}/K_m$  for the 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.<sup>295</sup> At pH 6.0 and 25 °C, RNase A catalyzes the transphosphorylation of UpA with a  $k_{\text{cat}}/K_m$  of  $2.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>146</sup> Under identical conditions, 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  $5 \times 10^{-9} \text{ s}^{-1}$  (which corresponds to  $t_{1/2} = 4 \text{ y}$ ).<sup>156</sup> The dissociation constant for the rate-limiting transition state during the transphosphorylation of UpA is therefore  $K_{\text{TX}} = k_{\text{uncat}}/(k_{\text{cat}}/K_m) = 2 \times 10^{-15} \text{ M}$ . Because the rate-limiting transition state may not involve a change in covalency,<sup>156</sup> 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.

What is the origin of the affinity of RNase A for the chemical transition state? Replacing Lys41 with an alanine residue removes a potential hydrogen-bond donor from the active site of RNase A. It is the ability of this residue to donate a hydrogen bond that enhances catalysis.<sup>155</sup> The loss of a hydrogen bond from residue 41 costs the enzyme 10<sup>5</sup>-fold in rate acceleration. Similarly, replacing His12 or His119,



**Figure 9.** Free energies for the uncatalyzed (---) and RNase A-catalyzed (—) transphosphorylation of UpA (left) and hydrolysis of U>p (right). 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  $k_{\text{cat}}/K_m$ <sup>146</sup> and  $k_{\text{cat}}$ <sup>156</sup> for UpA transphosphorylation,  $k_{\text{cat}}/K_m$  for U>p hydrolysis,<sup>37</sup> and  $k_{\text{uncat}}$  for C>p hydrolysis.<sup>297</sup> The free energy of uridine 3'-phosphate (3'-UMP) relative to that of U>p was calculated for their reaction at pH 6.0 and 25 °C with the equation:  $\Delta G^\circ = -RT \ln K$ , where  $K = 1.0 \times 10^3$ .<sup>233</sup> The free energies for the RNase A-catalyzed reactions are drawn for a standard state of 0.1 mM, which is the concentration of RNase A in the bovine pancreas.<sup>2</sup> The uncatalyzed hydrolysis of U>p also produces uridine 2'-phosphate in a reaction that is not shown.

the base and acid in catalysis (Figure 5), slows catalysis by 10<sup>4</sup>- to 10<sup>5</sup>-fold.<sup>154</sup> Finally, the B2 subsite of RNase A is also significant contributor to catalysis. This subsite, which interacts with the base of the residue that is part of the scissile phosphodiester bond, is composed of Asn71 and Glu111 (Figure 2).<sup>141</sup> The values of  $k_{\text{cat}}/K_m$  for the RNase A catalyzed transphosphorylation of substrates with different leaving groups decrease in the order: adenosine > guanosine > cytidine > uridine > methanol.<sup>16</sup> CpA is transphosphorylated by RNase A with  $k_{\text{cat}}/K_m = 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ; CpOMe with  $k_{\text{cat}}/K_m = 250 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>296</sup> If CpA interacts most strongly with the B2 pocket and CpOMe does not interact at all, then the binding of adenosine to the B2 subsite provides a 10<sup>4</sup>-fold rate acceleration. Thus, four factors (Lys41, His12, His119, and the B2 subsite) individually contribute at least 10<sup>4</sup>-fold in rate enhancement. Because the overall rate enhancement is  $3 \times 10^{11}$ , these factors cannot contribute independently to catalysis.

The free energies for the two steps in the hydrolysis of RNA can be derived from available data (Figure 9<sup>233,297</sup>).<sup>156</sup> At pH 6.0 and 25 °C, the intrinsic kinetic barrier for cleaving a P–O<sup>5'</sup> bond in RNA is almost identical to that for hydrolyzing the P–O<sup>2'</sup> or P–O<sup>3'</sup> bond in a nucleotide 2',3'-cyclic phosphodiester. Apparently, the proximity of the 2'-hydroxyl group to the phosphorus atom in RNA and the strain<sup>298–301</sup> (or poor solvation<sup>302</sup>) inherent in a nucleotide 2',3'-cyclic phosphodiester contribute equally to an enhanced rate of decomposition. These phosphodiester bonds are far less stable than are those in DNA, which suffer cleavage at a  $3 \times 10^4$ -fold lower rate.<sup>303</sup> Together, kinetic data on the cleavage of the P–O<sup>5'</sup> bond in RNA<sup>156</sup> and DNA<sup>303</sup> reveal that each proximal 2'-hydroxyl group of RNA has an effective concentration of  $2 \times 10^6 \text{ M}$  ( $= 3 \times 10^4 \times 55 \text{ M}$ ).

## X. Ribonuclease S

The protease subtilisin prefers to cleave a single peptide bond in native RNase A.<sup>304,305</sup> The product of this cleavage, ribonuclease S (RNase S, where "S" refers to subtilisin), consists of two tightly associated fragments. These fragments are S-peptide, which derives from residues 1–20 of RNase A, and S-protein, which derives from residues 21–124. Although neither fragment alone has any ribonucleolytic activity, RNase S has enzymatic activity similar to that of intact RNase A. The three-dimensional structure of crystalline RNase S<sup>306–308</sup> was determined soon after that of RNase A.<sup>58</sup> Because initial reports on the structure of RNase A lacked detail<sup>58</sup> (or were altogether incorrect<sup>309</sup>) early structural work on RNase S<sup>306,307</sup> greatly stimulated interest in the enzyme.<sup>310</sup> The structures of RNase S with bound uridylyl(3'→5')-5'-deoxy-5'-methyleneadenosine,<sup>311</sup> 2'-deoxy-2'-fluoro-UpA,<sup>312</sup> ApC,<sup>313</sup> and cytidylyl(2'→5')-adenosine<sup>314</sup> are also known.

### A. S-Protein–S-Peptide Interaction

Only a low yield of native S-protein is isolable from the air oxidation of reduced S-protein.<sup>88</sup> The recovery of native S-protein is complete, however, if the oxidation is performed in the presence of S-peptide, which presumably serves as a template for proper folding.<sup>315</sup> A monoclonal antibody against native S-protein has been shown to have a similar effect, enhancing by 3.6-fold the yield of native S-protein.<sup>316</sup> (In contrast to S-protein, the S-peptide portion of RNase A is not antigenic.<sup>317</sup>)

In addition to structural information, extensive thermochemical data have been acquired on the S-protein–S-peptide interaction. The value of  $K_d$  for RNase S is dependent on pH (ranging from  $3.1 \times 10^{-11}$  M at pH 8.3 to  $1.1 \times 10^{-6}$  M at pH 2.7<sup>318</sup>), temperature (ranging from  $8.3 \times 10^{-8}$  M at 30 °C to  $9.2 \times 10^{-6}$  M at 45 °C<sup>319</sup>), and ionic strength (increasing 7-fold as the concentration of NaCl is decreased from 0.5 M to 0.7 mM<sup>320</sup>). A complex of S-protein with only the 15 *N*-terminal amino acid residues of S-peptide (S15) is essentially identical in structure to that of RNase S.<sup>235</sup> Isothermal titration calorimetry has shown that the value of  $K_d$  for the S-protein·S15 complex is  $1.1 \times 10^{-7}$  M at 25 °C in 50 mM sodium acetate buffer, pH 6.0, containing NaCl (0.10 M).<sup>321</sup>

### B. New Technology

The S-peptide fragment of RNase A has had a singular role in the development of protein chemistry. Before molecular biologists were able to use recombinant DNA techniques to explore protein structure–function relationships, chemists synthesized analogues of S-peptide and studied their complexes with S-protein. The preparation of RNase S<sup>322–326</sup> by total synthesis occurred simultaneously with that of RNase A.<sup>11,71</sup> In addition, work on RNase S provided the first three-dimensional structure of a protein–nucleic acid complex,<sup>327</sup> as well as the first demonstration that a crystalline enzyme could be an active catalyst.<sup>328</sup> (For reviews, see refs 15 and 16. For histori-

cal accounts, see refs 329 and 330.) These studies were successful in illuminating molecular aspects of enzymatic catalysis, protein–protein interactions, and protein–nucleic acid interactions, and were the harbinger of current work on proteins containing variant or nonnatural amino acid residues. Work on the structure and function of another semisynthetic ribonuclease, RNase-(1–118)·(111–124),<sup>262,263</sup> has also made significant contributions.<sup>165,264,266,267,331–337</sup> Recently, the RNase S system has spawned or at least facilitated the development of many innovative technologies.

#### 1. Substrate–Leash Amplification

Chemical amplification takes place when a small chemical stimulus is magnified into a large chemical response.<sup>338</sup> The RNase S system has provided the first example of one type of chemical amplification: "substrate–leash amplification".<sup>339,340</sup> Here, the S-peptide or S-protein fragment is immobilized on solid supports via a "leash" of poly(C) substrate. Each support releases its fragment when treated with the complementary enzyme fragment or with RNase A. The fragments released from a mixture of the two supports recombine to give RNase S activity. This system provides an amplification of activity that exceeds  $10^4$ -fold. Such a cascade could serve as the basis for effective biosensors.

#### 2. Sequence-Specific Ribonuclease

RNase S has been engineered to cleave only a specific sequence in an RNA molecule.<sup>341</sup> This enhanced specificity is attained by attaching a thiol-modified DNA oligonucleotide to the *N*-terminal cysteine residue of K1C S-peptide via a disulfide bond. The synthetic construct allows for the formation of a hybrid RNase S that cleaves RNA with a specificity dictated by the DNA sequence. Analogous experiments have been performed with intact K1C RNase A.<sup>36,268,342</sup>

#### 3. Fusion Protein System

RNase S has served as the basis for a fusion protein system. Recombinant DNA technology has been used to produce a fusion protein in which S-peptide or S15 (also known as "S-TAG") is attached covalently to a target protein.<sup>186,343–345</sup> The interaction of the S-peptide portion of the fusion protein with immobilized S-protein allows for the facile purification of the fusion protein. Likewise, the interaction with soluble S-protein enables a sensitive ribonucleolytic assay to be used to detect the fusion protein either in solution<sup>186,343–345</sup> or after electrophoresis in a polyacrylamide gel.<sup>186,345</sup>

#### 4. Antagonist from Phage Display

The RNase S system has produced a notable success in combinatorial chemistry. S-protein has been used to pan a filamentous phage library displaying hexapeptides of random sequence.<sup>346</sup> The selected peptides have a sequence motif of (F/Y)NF-(E/V)(I/V)(L/V), which bears little resemblance to the sequence of S-peptide. One of the displayed peptides,

YNFEVL, binds to S-protein to form a complex with a  $K_d$  of  $5.5 \times 10^{-6}$  M, which is comparable to that of RNase S. The S-protein·YNFEVL complex has no detectable enzymatic activity, so the phage display system has revealed a true antagonist.

### 5. Semisynthetic Transaminases

The architecture of an enzyme can be used to modulate an unrelated chemical reaction.<sup>347</sup> The architecture of RNase S has been shown to enhance a transamination reaction. In a variety of semisynthetic enzymes, Phe8 of C-peptide (which encompasses residues 1–14<sup>348</sup>) has been replaced with a coenzyme–amino acid chimera containing a pyridoxal<sup>349,350</sup> or pyridoxamine<sup>351</sup> side chain. The resulting complexes with S-protein increase (albeit modestly) the rate and enantioselectivity of the conversion of an  $\alpha$ -amino acid to an  $\alpha$ -keto acid, or vice versa.

### 6. Protein Ubiquitination

RNase S has been used to explore the specificity of protein ubiquitination.<sup>352</sup> A fusion between S15 and the ubiquitin-conjugating enzyme E2 directs crude cell extracts to attach ubiquitin to S-protein. This result demonstrates that a target protein can be ubiquitinated (and thereby fated for degradation) simply by appending an appropriate interaction domain onto a ubiquitin-conjugating enzyme.

### 7. Protein–Protein Interactions

The RNase S system has been used to demonstrate the utility of green fluorescent protein (GFP) in the revelation and characterization of protein–protein interactions.<sup>353</sup> Recombinant DNA technology has been used to produce a fusion protein in which S15 is attached covalently to a GFP variant that resembles fluorescein in its excitation and emission wavelengths. The interaction of this fusion protein with S-protein has been analyzed by two distinct methods: fluorescence gel retardation and fluorescence polarization. The fluorescence gel retardation assay is a rapid method to reveal a protein–protein interaction and to estimate the  $K_d$  of the resulting complex. The fluorescence polarization assay is an accurate method to evaluate  $K_d$  in a homogeneous solution and can be adapted for the high throughput screening of protein or peptide libraries.<sup>354</sup>

## XI. Molecular Evolution

The amino acid sequences of proteins that are homologous (that is, have a common evolutionary origin<sup>355</sup>) often vary between different species of organisms. This variation results from both selective adaptation<sup>356</sup> and neutral drift.<sup>357,358</sup> The conservation (or divergence) of particular amino acid residues in homologous proteins can lend support to experimental findings as well as provoke new questions about protein structure and protein function. In addition, sequence data enables the reconstruction of the evolutionary history of a protein. This reconstruction is done by applying parsimony analysis<sup>359</sup> to the aligned amino acid sequences. The result is a

phylogenetic tree that predicts the amino acid sequences in ancestral organisms.<sup>360</sup>

The amino acid sequences of RNase A homologues have been obtained from over 40 different vertebrates. (For reviews, see refs 20, 22, and 361.) RNase A has thus become a model system for elaborating the consequences of molecular evolution in vertebrate taxa. From these sequences and their organismal distribution, it is apparent that RNase A is a modern protein that is evolving rapidly.<sup>362</sup> Using site-directed mutagenesis, several putative ancestors of RNase A have been produced to address issues in the evolution of vertebrate physiology.<sup>363,364</sup> The evolutionary reconstruction of artiodactyl homologues of RNase A has been the subject of a recent review.<sup>365</sup>

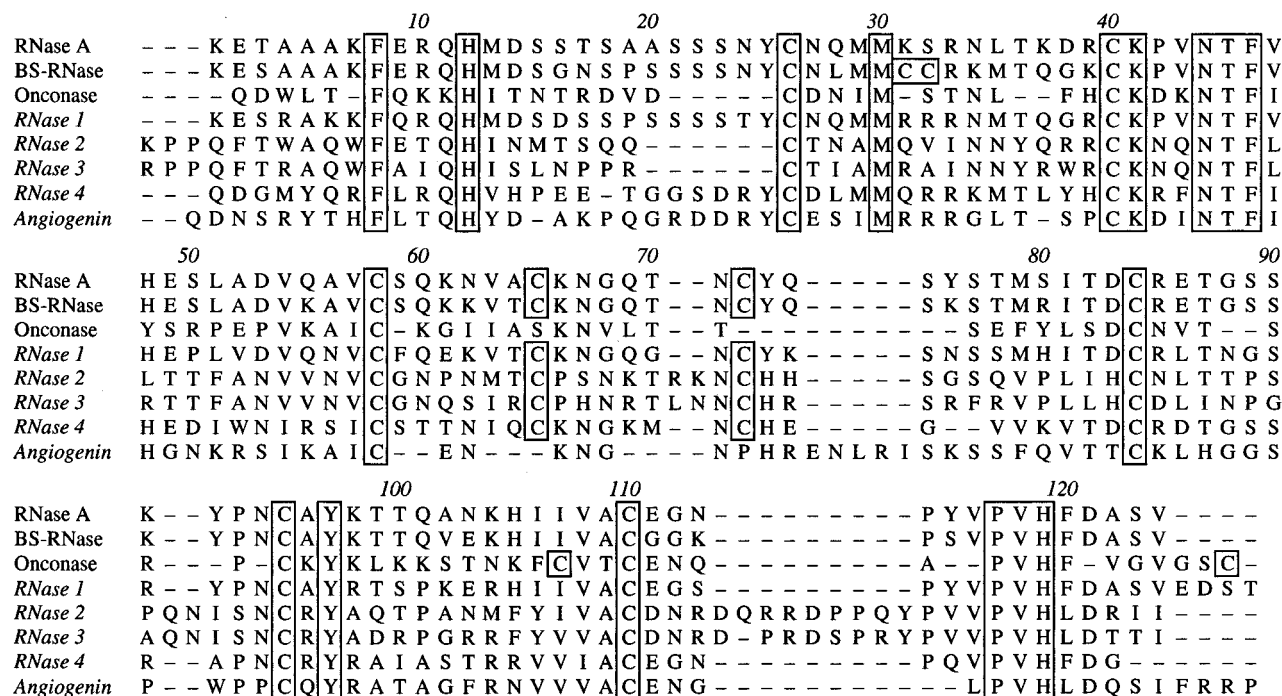
## XII. Unusual Homologues

The functions typically ascribed to ribonucleases are to process and turnover cellular RNA and to degrade dietary RNA. Yet, some homologues of RNase A appear to have quite different biological roles. (For general reviews, see refs 366 and 367.) These homologues were discovered on the basis of their unusual activities. Only later, sometimes much later, were the proteins identified as ribonucleases.

Ribonucleases can be cytotoxic because cleaving RNA renders indecipherable its encoded information (eq 1). The cytotoxicity of ribonucleases was discovered in the 1950s. RNase A was shown then to be toxic to tumor cells, both in vitro<sup>368</sup> and in vivo.<sup>369,370</sup> Large doses of RNase A were used in these early studies—effects were observed only after milligrams of enzyme were injected into solid tumors. Subsequently, smaller doses of RNase A were found to have no effect.<sup>371</sup>

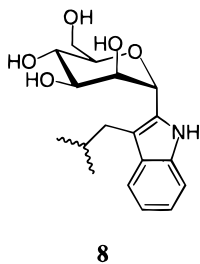
Over 20 years ago, a homologue of RNase A was discovered in bull seminal plasma that is cytotoxic at low levels.<sup>372,373</sup> In the past decade, even more cytotoxic homologues were isolated from the eggs of the bullfrog *Rana catesbeiana*,<sup>374,375</sup> the Japanese rice paddy frog *Rana japonica*,<sup>376</sup> and the Northern leopard frog *Rana pipiens*.<sup>377</sup> All of these *Rana* ribonucleases are toxic to tumor cells in vitro with  $IC_{50}$  values near 1  $\mu$ M.<sup>378,379</sup> The mechanism of cytotoxicity could involve binding to cell-surface glycolipids, retrograde transport to the Golgi apparatus<sup>380</sup> and the endoplasmic reticulum, translocation into the cytosol, and degradation of cellular RNA.<sup>381</sup> A key to this last step is the evasion of RI (see section VIII), which binds tightly to RNase A but not to its cytotoxic homologues.<sup>378,382–384</sup> Bovine seminal ribonuclease<sup>385,386</sup> and the *Rana* ribonucleases<sup>387</sup> have been the objects of recent reviews.

Humans contain at least five homologues of RNase A (Figure 10).<sup>395</sup> RNase 1 (which is from human pancreas)<sup>388,396</sup> and RNase 4 (which is from human liver)<sup>391,397</sup> are distinct enzymes. Angiogenin is a plasma enzyme that promotes neovascularization.<sup>389,398,399</sup> Eosinophilic leukocytes contain RNase 2 (eosinophil-derived neurotoxin; EDN), which is neurotoxic, and RNase 3 (eosinophil cationic protein; ECP) which has helminthotoxic and antibacterial activities.<sup>400</sup> Human urine and human erythrocytes



**Figure 10.** Amino acid sequences of RNase A,<sup>52,53</sup> two cytotoxic homologues (bovine seminal ribonuclease<sup>390</sup> and onconase<sup>377</sup>), and five human homologues (RNase 1,<sup>388</sup> RNase 2,<sup>391,393</sup> RNase 3,<sup>392,394</sup> RNase 4,<sup>395</sup> and angiogenin<sup>389</sup>). The sequences were aligned by using the program PILEUP (Genetics Computer Group—Version 9; Madison, WI) with GapWeight 1.000 and GapLengthWeight 0.100. Boxes are drawn around cysteine residues and completely conserved residues.

contain a protein that is identical to RNase 2, with residue 7 being an unusual C<sup>2</sup>- $\alpha$ -mannosyltryptophan (**8**).<sup>401–403</sup> RNase 1,<sup>42,404</sup> RNase 2,<sup>404,405</sup> RNase 3,<sup>406</sup> RNase 4,<sup>404</sup> and angiogenin<sup>198,407</sup> are all bound tightly by human RI. Angiogenin<sup>408</sup> and the eosinophilic ribonucleases<sup>409,410</sup> have been the objects of recent reviews.



The crystalline structures of several homologues of RNase A have been determined by X-ray diffraction analysis. These homologues include bovine seminal ribonuclease,<sup>411,412</sup> *Rana pipiens* ribonuclease (which is also known as onconase),<sup>413</sup> human angiogenin,<sup>414</sup> bovine angiogenin,<sup>415</sup> and human RNase 2.<sup>416</sup> The crystalline structures of RI<sup>200</sup> and an RI·RNase A complex,<sup>201,202</sup> which is not ribonucleolytic, are also known.

All of the RNase A homologues described above catalyze the cleavage of RNA. Wild-type levels of ribonucleolytic activity are essential for the cytotoxic and other biological activities of bovine seminal ribonuclease,<sup>417</sup> onconase,<sup>377</sup> RNase 2,<sup>404</sup> and angiogenin.<sup>418</sup> In surprising contrast, the wild-type ribonucleolytic activity of RNase 3 is not essential for its antibacterial activity.<sup>419</sup> Revealing the mechanism by which the ribonucleolytic activity of these RNase A homologues is manifested in their unusual biologi-

cal activities is now a fruitful area for research that spans the chemistry–biology interface.<sup>420</sup>

### XIII. Conclusion

RNase A has been the most studied enzyme of the 20th century. Methods now exist to produce unlimited quantities of RNase A (and its homologues) in which any amino acid residue is changed to any other. Methods also exist to synthesize informative and useful nucleotides and nucleotide analogues. These methods leave us poised to reveal in even more detail the precise role of enzymic residues, and to exploit further the use of RNase A in biotechnology and medicine. Work on RNase A will continue to provide a chemical framework for work on other ribonucleases, on ribozymes, and on synthetic catalysts of nucleic acid cleavage.

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