# Role of the His---Asp Catalytic Dyad of Ribonuclease A

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To my family

#### ABSTRACT

Hydrogen-bonded histidine and aspartate residues are found in the active sites of many proteases, lipases, and ribonucleases. We have used a variety of methods to probe the role of the catalytic dyad of bovine pancreatic ribonuclease A (RNase A). In RNase A, His119 and Asp121 form this dyad. Our work relied largely on the analysis of mutant enzymes in which Asp121 was replaced by an asparagine or alanine residue, or in which His119 was replaced by an alanine residue.

Steady-state kinetic analyses revealed that Asp121 has a nominal role in catalysis. Our data also showed that the two conformations occupied by His119—one in which it can interact with Asp121 and another, brought about through rotation about the  $C_{\alpha}$ —C $\beta$  bond, in which it cannot—have similar catalytic activities. The pH-dependence of  $k_{cat}$  and  $K_m$  for the hydrolysis of uridine 2',3'-cyclic phosphate revealed an alternative pathway that depends on a group with a p $K_a$  of 5.0. Evidence was also found for a residue with a p $K_a$  of 3.8, which must be unprotonated to bind substrate. We postulate that this p $K_a$  arises from Asp83.

<sup>1</sup>H-NMR titration curves showed that Asp121 has a minimal effect on the  $pK_a$  of His119. In addition, titrations of the unliganded enzymes and the enzymes in the presence of two concentrations of the inhibitor uridine 3'-phosphate revealed a second molecule of inhibitor binds near the catalytic dyad.

Surprisingly, the contribution of Asp121 to protein stability is more significant than its contribution to enzymatic catalysis. pH-Dependences of the unfolding transitions indicated that the  $pK_a$ 's of Asp121 in RNase A in the native and the denatured states were 2.7 and 3.6, respectively, and that His119 is largely responsible for this decrease in the  $pK_a$  of Asp121. Compensating enthalpy – entropy changes, possibly due to the loss of a hydrogen bond between Ala121 and Lys66, resulted in equivalence in the stability of D121N and D121A

RNase A. The two conformations of His119 were calculated to have  $K_{eq} = 2.5$  (pH 1.2) in favor of the interaction with Asp121.

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## Abbreviations

RNase A	bovine pancreatic ribonuclease
AcOH	acetic acid
AMPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-
	hydroxypropanesulfonic acid
BICINE	N,N-bis(2-hydroxyethyl)-glycine
BIS-TRIS	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-
	propanediol)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
EDTA	ethylenediaminetetraacetate
TE	Tris/HCl EDTA
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
poly(C)	poly(cytidylic acid)
UpA	uridylyl(3' $\rightarrow$ 5')adenosine
cUMP	uridine 2',3'-cyclic phosphate
cCMP	cytidine 2',3'-cyclic phosphate
3'-CMP	cytidine 3'-phosphate
3'-UMP	uridine 3'-phosphate.
3'-dCMP	2'-deoxycytidine 3'-phosphate

2'-AMP	adenosine 2'-phosphate
3'-AMP	adenosine 3'-phosphate
5'-AMP	adenosine 5'-phosphate
C <sub>x</sub>	C <sub>x</sub> atom of histidine
pH*	uncorrected pH meter reading of a D2O solution made
	with a glass electrode standardized in H <sub>2</sub> O buffers.

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# Chapter 1

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Introduction: The Role of the His…Asp Catalytic Dyad of Ribonuclease A Although the catalytic machinery used by enzymes is wide and varied, most enzymes fall into classes wherein great similarities exist. One well-studied class is that of the serine proteases. This class of enzymes displays a catalytic motif known as the catalytic triad, which is composed of the residues Ser•His•Asp linked together by hydrogen bonds in this respective order. The importance ascribed to this motif is in no small measure due to the fact that it is present in two families, the "trypsin-like" and the "subtilisin-like", and possibly even a third family referred to as "nonclassical" (Frederic *et al.*, 1993), that appear to have evolved this motif independently. In recent years, this triad has been found in yet another class of enzymes, the lipases (Brady *et al.*, 1990; Winkler *et al.*, 1990) (Although at least in one lipase, the aspartate residue is replaced by a glutamate (Schrag *et al.*, 1991)). Here also the motif appears to have evolved independently.

A similar motif is observed in phospholipase  $A_2$ , pancreatic ribonucleases, and in many proteins related to the latter and known as the ribonuclease superfamily. Here, the hydroxyl of the serine residue is replaced by the hydroxyl of a substrate. This His•Asp motif is sometimes referred to as the "catalytic dyad". This dyad is also found in the zinc enzyme family as the triad Asp•His•Zn<sup>2+</sup> where it has been speculated to be related to the catalytic triad/dyad (Christianson & Alexander, 1989). In serine proteases, lipases, and pancreatic ribonucleases, two reactions are catalyzed in which a hydroxyl group from either the enzyme or the substrate displaces a group on the substrate and is itself displaced by a molecule of water. In phospholipase  $A_2$ , hydrolysis alone occurs.

In all enzymes containing a catalytic triad/dyad, the hydroxyl group is believed to function as a nucleophile, and the role of the histidine residue is abstraction of the hydroxyl group's proton so as to increase its nucleophilicity. The aspartate residue, in turn, is postulated to aid histidine in some form or another. Just what form this "aid" takes has been the subject of much study and debate, as has the role of the catalytic triad in general (for a review see (Schowen, 1988)).

The catalytic triad was once thought to act in the "charge relay mechanism". Specifically, it was postulated that a proton was removed from the serine residue by histidine while another proton was removed from histidine by aspartate (Blow *et al.*, 1969). The general consensus today is that the charge relay mechanism is not operative, as the  $pK_a$  of the aspartate is too low to favor such a mechanism. Not all, however, are convinced of this (Umeyama *et al.*, 1979). It can be argued that the  $pK_a$  in the transition state differs from that of the ground state, and thus aspartate may be involved in a very fleeting proton transfer (Schowen, 1988). The fact that the transition state is indeed so fleeting, of course, makes it difficult, though not impossible, to prove or disprove such a notion. Still others have argued for the presence of a low barrier hydrogen bond between histidine and aspartate that might impart a rate increase of approximately 104 fold (Frey *et al.*, 1994). More conservative arguments hold that the negatively charged aspartate electrostatically stabilizes the formation of the imidazolium ion or that its role is that of stabilizing the orientation of histidine via a hydrogen bond.

Two groups have attempted to resolve the question of the role and importance of aspartate in the catalytic triad of the serine proteases by substituting it with asparagine in trypsin (Craik *et al.*, 1985; Sprang *et al.*, 1987) or alanine in subtilisin (Carter & Wells, 1988) using site-directed mutagenesis. They both observed a reduction in rate of approximately 10<sup>4</sup> fold. This decrease would seem to argue for a very important role for aspartate. However, structural studies of the trypsin mutant revealed that the catalytic histidine was being stabilized as an unproductive tautomer through its interaction with asparagine. Although this result argues for a role of aspartate in orienting histidine, it does not explain why evolution did not use any of a number of other ways of doing the same.

Two similar attempts have been made to determine the role of aspartate in the catalytic dyad of bovine pancreatic ribonuclease A (RNase A). In one study, Asp121, the aspartate residue of the catalytic dyad, was replaced with asparagine in a semisynthetic enzyme (Stern

& Doscher, 1984). This semisynthetic RNase, RNase-(1-118)\*(111-124), consists of a noncovalent complex between residues 1-118 of RNase (obtained from proteolytic digestion of RNase A), and a synthetic peptide composed of the 14 carboxyl-terminal residues of RNase A, except with Asp121 replaced by an asparagine residue. The D121N semisynthetic mutant was observed to have approximately 5% of the activity of the analogous wild-type semisynthetic enzyme.

A potential difficulty with such a system, however, is that the semisynthetic enzyme may have structural deviations from that of a covalently linked complex. Indeed, although wild-type RNase A and its semisynthetic analog appear to have very similar structures (Martin *et al.*, 1987), that of the D121N enzyme exhibits numerous changes (deMel *et al.*, 1992). Most notably, the loop containing residues 65-72 moves away from the active site. Preliminary studies in this lab indicate that the crystal structures of D121N and wild-type RNase A are indistinguishable (Schultz *et al.*, 1995). Furthermore, the differential effects of the D121N mutant on the  $pK_a$ 's of the active-site histidine residues also suggests that structural differences accompany semisynthesis (see Chapter 3 and (Cederholm *et al.*, 1991)).

In another study, Asp121 was replaced with a glutamate residue using site-directed mutagenesis (Trautwein *et al.*, 1991). The catalytic activity of the mutant enzyme was approximately 17% that of wild type. Lacking more information about this mutant enzyme, such as its effect on the  $pK_a$  of the histidine of the catalytic dyad, its effect on stability, or its structure, we are not much the wiser as to the role of aspartate in the dyad.

Here, we have attempted to address the role of the aspartate residue in the catalytic dyad of bovine pancreatic ribonuclease A (RNase A) by replacing it with asparagine and alanine residues via site-directed mutagenesis and observing the effect these mutations have on catalysis, stability, and on the  $pK_a$ 's of the active site histidines—in particular the one in the catalytic dyad.

Bovine pancreatic ribonuclease (EC 3.1.27.5) was chosen for this study as it is well characterized in many respects, thus allowing us to interpret more rigorously our results (Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Wlodawer, 1985; Eftink & Biltonen, 1987; Beintema *et al.*, 1988). This characterization includes structure, stability, folding pathways, protein chemistry, enzymology, and molecular evolution.

RNase A specifically catalyzes the two-step hydrolysis of the P–O<sub>5</sub> bond of RNA after pyrimidine residues. Figure 1.1 depicts the consensus mechanism for these reactions. In the transphosphorylation step, His12 functions as a general base and His119 as a general acid. The slow hydrolysis of the 2',3'-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation. The catalytic dyad is composed of the residues His119 and Asp121. These are conserved across the 41 pancreatic ribonucleases of known sequence, in bovine seminal ribonuclease, in nonsecretory RNase, and in the distantly related enzyme angiogenin (a stimulator of blood vessel formation) (Beintema, 1987; Beintema *et al.*, 1988). Of approximately 124 residues found in these proteins, only 21 are conserved completely. These include 6 half-cystine residues involved in disulfide bonds, the active-site residues His12 and Lys41, and the catalytic dyad.

The observation of conservation is strongly suggestive of the importance of these residues to the enzymes' function. Barnard has argued that pancreatic ribonucleases are "essential only in ruminants and certain other herbivores" and thus "can be expected to show wide molecular changes in evolution" (Barnard, 1969). Hence, the conservation—ranging from turtles to whales—is all the more suggestive of the significance of the function of these residues.

In Chapter 2 we have demonstrated that the mutant enzymes have suffered a relatively nominal decrease in catalytic activity, suggesting a relatively minor role for Asp121 in catalysis. This observation seems to belie the evolutionary arguments made above. In Chapter 3 we have shown that the effect of Asp121 on the  $pK_a$  of His119 is surprisingly small. Thus, the argument that the role of aspartate in the dyad is that of perturbing the  $pK_a$  of His119 towards that needed for maximal activity does not appear to be justified here.

In Chapter 4, however, we have found that the contribution that Asp121 makes to stability is significant. Much of this contribution appears to be due to electrostatic interaction between Asp121 and His119. It is conceivable that all of the variance is electrostatic as the difference in stability of Asp121 and Asn121 at very acidic pH where Asp121 is protonated may be due to a favorable hydrogen bond made by Asp121, either to His119 or Lys66, versus that made by Asn121.

Finally, we would like to consider the possibility that part of the role of Asp121 may be that of a more biological nature. RNase A may have biological properties in which the loss of Asp121 may be detrimental. RNase A is known to inhibit cancer cell growth in culture and to prolong survival in animal tumor models (Roth, 1963). This might keep up the evolutionary pressure for maximal activity and stability. Proteins related to RNase A have been shown to have diverse biological properties (Leone *et al.*, 1973; Beintema *et al.*, 1988). Conceivably RNase A may have some as yet undiscovered biological activity.

A study done by Vallee and coworkers is suggestive of the possible role Asp121 may have in biological activity (Curran *et al.*, 1993). They replaced Asp116 of angiogenin (the functional counterpart of Asp121 in RNase A) with a number of different residues and observed changes in enzymatic activity as well as angiogenic activity. They postulated that, in addition to modulating catalysis, Asp116 maybe involved in receptor binding by communication with the loop residues 62-71 (65-72 in RNase A), the putative cell-surface receptor binding site implicated as being necessary for angiogenic activity. Figure 1.1 Mechanism of the transphosphorylation (top) and hydrolysis (bottom) reactions catalyzed by RNase A (Findlay et al., 1961). In the hydrolysis reaction, the hydrogen bonds between Asp121...His119...H<sub>2</sub>O resemble those in the renown catalytic triad of proteases.

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# Chapter 2

His---Asp Catalytic Dyad of Ribonuclease A: Effect of pH on Catalysis by the Wild-Type, D121N, and D121A Enzymes ABSTRACT Hydrogen-bonded histidine and aspartate residues are found in the active site of many proteases. lipases, and ribonucleases. Here, Asp121 of the catalytic dyad of bovine pancreatic ribonuclease A was replaced with asparagine or alanine residues. The steady-state kinetic parameters for catalysis by these mutant enzymes was determined for the cleavage of uridylyl(3' $\rightarrow$ 5')adenosine and poly(cytidylic acid), and the hydrolysis of uridine 2',3'-cyclic phosphate (cUMP). The results of this analysis argue that the two conformations occupied by the histidine of the dyad (His119)—one in which it can interact with Asp121 and another, brought about through rotation about the C $\alpha$ -C $\beta$  bond, in which it cannot—have similar activity. The pH-dependence of the parameters for cUMP hydrolysis was also determined. The primary effect of these mutations is to reduce the value of  $k_{cat}$  by 3- to 50-fold. The pHdependence of  $k_{cat}$  and  $K_m$  for the substrate cUMP revealed an alternative pathway that depended on a group with a  $pK_a$  of 5.0. Evidence has also been found for a residue with a  $pK_a$  of 3.8 which must be unprotonated to bind substrate. We postulate that this  $pK_a$  arises from Asp83.

#### INTRODUCTION

Bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) is one of the most extensively studied enzymes (Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Wlodawer, 1985; Eftink & Biltonen, 1987; Beintema *et al.*, 1988). Previous studies include investigations of structure, stability, folding pathways, protein chemistry, enzymology, and molecular evolution; many of which are landmark works. Yet questions, such as what are the precise roles of the active-site residues in catalysis, still remain.

The active sites of several classes of enzymes—proteases, lipases, and pancreatic ribonucleases—contain a histidine residue hydrogen bonded to an aspartate. This motif is referred to as the catalytic triad in lipases and serine proteases (where serine is the third residue). Here, we refer to the His•••Asp pair in pancreatic ribonucleases as the catalytic dyad. All of these enzymes catalyze two reactions in which a hydroxyl group from either the enzyme or the substrate attacks and displaces a group on the substrate followed by hydrolysis at the same center with the regeneration of the hydroxyl group. A recent study arguing that the aspartate in the catalytic triad of the serine protease chymotrypsin partakes in a low-barrier hydrogen bond with the histidine is illustrative of the questions remaining regarding the function of this theme (Frey *et al.*, 1994).

The motif is completely conserved but for one lipase in which the aspartate is replaced by a glutamate (Schrag *et al.*, 1991). Due to a lack of structural similarity between ribonucleases, serine proteases, lipases, and even within the family of the serine proteases, it has been argued that this motif is an example of convergent evolution (Blow, 1990). The observation of conservation combined with the convergent evolution argument is strongly suggestive of the importance of these residues to the enzymes' functions. Barnard has argued that pancreatic ribonucleases are "essential only in ruminants and certain other herbivores" and thus "can be expected to show wide molecular changes in evolution" (Barnard, 1969). Hence, the conservation across the 41 pancreatic ribonucleases of known sequence—ranging from turtles to whales—is all the more suggestive of the significance of the function of these residues (Beintema, 1987).

In RNase A, the catalytic dyad is composed of residues His119 and Asp121. We have examined the catalytic role of Asp121 by replacing it with an alanine or asparagine residue. The relatively nominal decrease in activity we observe for the D121N mutant seems to belie the evolutionary arguments made above. Instead, the conservation may have root in other forces such as stability, resistance to proteolytic degradation, or biological properties such as those seen with a number of distantly related proteins (Beintema *et al.*, 1988).

#### MATERIALS AND METHODS

#### Materials

*E. coli* strain BL21(DE3) (F<sup>-</sup> ompT r<sub>B</sub>-m<sub>B</sub>-) (Studier & Moffatt, 1986) was from Novagen (Madison, WI). Buffers (except Tris), cUMP, and IPTG were from Sigma. Tris was from Fisher. Poly(C) was from Midland Reagent (Midland, TX). UpA was synthesized by J. E. Thompson using the methods of Ogilvie (Ogilvie *et al.*, 1978), and Beaucage and Caruthers (Beaucage & Caruthers, 1981). Growth media were from Difco or, for the 10L growths, Marcor Development (Hackensack, NJ). DNA sequences were determined with the Sequenase Version 2.0 kit from U.S. Biochemicals. DEAE Sephadex A-25 anion exchange resin, S-Sepharose cation exchange resin, and the mono-S column were from Pharmacia LKB. Bacterial terrific broth (TB) contained (in 1 L) tryptone (12 g), yeast extract (24 g), glycerol (4 mL), KH<sub>2</sub>PO<sub>4</sub> (2.3 g), and K<sub>2</sub>HPO<sub>4</sub> (12.5 g).

#### Mutagenesis

Previously, we described the construction of a cow pancreatic cDNA library, the cloning of the cDNA that codes for RNase A, and the efficient expression of this cDNA in *Escherichia* 

coli (delCardayre et al., 1995). Here, the codon for Asp121 was changed to one for an asparagine or alanine residue by oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) using the oligonucleotides: 5' ACTACTACACGCTAGCGTTAAAGTGGACT 3' and 5' ACTACTACACGCTAGCGCTAGCGGCAAAGTGGACT 3'. The GCT mismatch introduced a unique, translationally silent NheI site that was used to screen for mutated plasmids. The complete cDNA of each protein was analyzed by sequencing. Plasmids that direct the expression of wild-type, D121N, and D121A RNase A were transformed into *E. coli* BL21(DE3) for expression.

#### Production of RNase A in E. coli

Briefly, 200 mL of TB broth containing ampicillin (200  $\mu$ g/mL) was inoculated with a culture frozen in 30% glycerol of the appropriate plasmid. After growth overnight at 37 °C, cells were collected by centrifugation and suspended in 200 mL of fresh TB. This suspension was used to inoculate 10 L of TB containing ampicillin (200  $\mu$ g/mL). When A = 4 at 600 nm, IPTG was added to a final concentration of 0.5 mM. The culture was grown for an additional 3.5 h.

Cells were collected by centrifugation, resuspended in 100 mL of cold TE buffer, and lysed by passage through a French pressure cell twice. The lysate was centrifuged at 30,000  $\times$  g for 30 min. The resulting pellet was washed with a cold solution (300 mL) of deoxycholate (1 mg/mL), and centrifuged again. The pellet was suspended in a 500 mL solution of Tris-HOAc buffer, pH 8.0, containing urea (9 M), sarcosine (40 mM), EDTA (1 mM), and 50 mM acetic acid (pH 8). Sonication was used to aid dissolution. To reduce the disulfide bonds, DTT was added to a final concentration of 50 mM. After 1 h, the solution was passed over 25 g of DEAE A-25, which had been equilibrated with the above resuspention solution, and 1/10th volume acetic acid added to the effluent. The effluent was dialyzed exhaustively against 20 mM acetic acid. The dialysate was centrifuged, the supernatant was diluted to 1 L, and the pH brought to 8.1 by the addition of Tris base. This solution was made 2.3 mM in reduced and 0.8 mM in oxidized glutathione. After 24 h the resulting solution was loaded on a S-Sepharose column (200 mL). The column was washed with 1 L of 50 mM HEPES buffer, pH 7.9, and the adsorbed products eluted with a linear gradient (500 mL + 500 mL) of NaCl (0 – 0.50 M). Fractions were pooled on the basis of absorbance at 280 nm, diluted with buffer, and loaded on to a mono-S cation exchange column (15 cm x 1.8 cm<sup>2</sup>) equilibrated with 50 mM HEPES buffer, pH 7.9. The column was run as per the suggestions of the manufacturer with no more than 10 mg of protein loaded per run. The proteins eluted at ~0.09 to 0.12 M NaCl. Fractions were again pooled on the basis of absorbance at 280 nm (monitored continuously). On account of the apparent deamination we observed for these proteins when held at alkaline pH, the pH of the solution was then adjusted to ~5.5. After exhaustive dialysis against water, the proteins were concentrated with a Amicon Centriprep-10 $\otimes$  filter, passed through a 0.45-µm filter (Scheicher & Schvell, Keene, NH), and stored at 4 °C.

The purity of the preparation was assessed by SDS-PAGE (Laemmli, 1970) and native polyacrylamide gels. Due to the base line resolution, symmetry of the peaks in the  $A_{280}$  chromatograph, and the presence of only one band on polyacrylamide gels, wild-type and the D121N mutant are estimated to be  $\geq$ 99% pure. Due to increased degradation of the D121A mutant, baseline resolution was not possible; the purity of this mutant is estimated to be  $\geq$ 95%.

#### **Enzyme Kinetics**

Steady-state kinetic parameters for the cleavage of UpA and poly(C), and the hydrolysis of cUMP were determined by spectrophotometric assays using a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. The reactions were assayed in a 100 mM solution of the appropriate buffer, adjusted with NaOH or HCl. Enough NaCl

was added to each reaction to increase the ionic strength to 0.10 M, which was calculated based on the concentrations of buffer components and substrate. Substrate concentrations were determined by ultraviolet absorption using  $\varepsilon_{260} = 24,600 \text{ M}^{-1}\text{cm}^{-1}$  at pH 7.0 for UpA (Warshaw & Tinoco, 1966) and  $\varepsilon_{268} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 7.8 for poly(C) (Yakovlev *et al.*, 1992) or by mass for cUMP. The buffers used for cUMP are give in Table 2.2. The concentrations for this substrate were generally from  $K_m/4$  to  $5 \times K_m$ , with a maximum concentration of 100 mM. The other substrates were assayed at pH 6.0 with MES buffer. These substrates were used at concentration ranges of 0.1 - 1.0 mM for UpA and 0.05 - 1.0 mM for poly(C). To the poly(C) reactions, 0.5 mg/ml BSA was added. All assays were performed at 25 °C in either a 1.0 or 0.2 cm cuvette.

The hydrolysis of cUMP was monitored at either 282 nm or 293 nm. The values for the extinction coefficients for the hydrolysis of cUMP are listed in Table 2.2. The cleavage of UpA was monitored at 286 nm. The  $\Delta \varepsilon$  was determined to be -755 M<sup>-1</sup>cm<sup>-1</sup> at pH 6.0. The cleavage of poly(C) was monitored at 238 nm. The  $\Delta \varepsilon$  for poly(C), calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product, has been reported to be 2380 M<sup>-1</sup>cm<sup>-1</sup> at pH 6.2 at 250 nm (Yakovlev *et al.*, 1992). A proportionality constant of 1.17 was applied to determine the  $\Delta \varepsilon$  at 238 nm as being 2792 M<sup>-1</sup>cm<sup>-1</sup> by observing the change in absorption of a partially cleaved substrate at these two wavelengths.

#### Data Analysis

Data processing and nonlinear regression analysis were performed using the program MATHCAD (MathSoft, Inc.; Cambridge, MA). Initial velocity data were weighted proportionately. The pH rate profiles were fit using the logarithm of the kinetic constants. Reported errors are generated by the asymptotic variance-covariance matrix method. Due to strong product inhibition and the relatively small extinction coefficient, the initial velocities

for the substrate cUMP were difficult to reliably measure. For this reason the steady-state kinetic parameters were determined by using the integrated rate equation. This also allowed for the determination of the product inhibition constant. Each progress curve for the hydrolysis reaction was fit to the integrated rate equation with the inclusion of product inhibition by an iterative procedure in which the kinetic parameters were varied by hand and the fit judged by eye. The value of  $k_{cat}$  was allowed to vary from progress curve to progress curve to allow for small errors in enzyme concentration, while the values of  $K_m$  and  $K_p$  (the product inhibition constant) were held constant. The derivative of the functions so obtained was used to determine improved values for the initial velocities. These velocities were then used to obtain the kinetic parameters using the Michaelis-Menten equation. If the parameters so realized differed greatly from the initial values, the process was repeated until they differed little from the prior round. Generally, one or two rounds were sufficient.

#### RESULTS

The kinetic constants for the reaction of three substrates, cUMP, UpA, and poly(C), are shown in Table 2.1. The mutations affect primarily  $k_{cat}$ , for the dinucleotide UpA, however, the mutants display an approximate 5-fold increase in  $K_m$  over that of the wild-type enzyme. For all three substrates, the  $k_{cat}$  for D121A RNase A is approximately  $\delta$ -fold lower than that of the D121N enzyme and 20- to 50-fold lower than that of wild-type RNase A.

Kinetic constants for the hydrolysis of cUMP at different values of pH are shown in Table 2.2. These data are plotted for  $k_{cat}/K_m$ ,  $k_{cat}$ , and  $K_p$  in Figures 2.1 – 2.3. For the D121A enzyme, data were not collected at the extremes of pH due to the large amounts of protein needed and a limited supply of the same. Also,  $k_{cat}$  is not reported at pH 9.0 due to an inability to achieve a sufficient amount of saturation.

In Figure 2.1, wild-type, D121N, and D121A RNase A are seen to have virtually identical  $k_{cat}/K_m$  profiles save for an alkaline dip in the profile of the D121N enzyme. Both wild-type and D121N RNase A display a second low pH limb at a pH = 4.

As shown in Figure 2.2, wild-type, D121N, and D121A RNase A have similar  $k_{cat}$  profiles at values of pH < 7, though that of D121N is somewhat shallower than that of wild-type. At values of pH greater than 7, the  $k_{cat}$  profile of the wild-type enzyme plateaus but those of the mutant enzymes' decreases. The  $k_{cat}$  profiles for wild-type and D121N RNase A have slopes of one at values of pH less than 4.5; all three proteins have slopes of much less than one between 4.5 and 7.0.

The  $K_p$  profile (Figure 2.3) of D121N RNase A is similar to that of the wild-type enzyme except for a decreased  $K_p$  at high pH. The  $K_p$  of D121A RNase A is twofold less than that of the other two proteins.

The solid lines in Figures 2.1 - 2.3 are drawn according to the following equations:

$$\frac{k_{\text{cat}}}{K_{\text{m}}} = \frac{(k_{\text{cat}} / K_{\text{m}})^{\text{int}}}{(1 + [\text{H}^+] / K_a)(1 + K_b / [\text{H}^+])(1 + [\text{H}^+] / K_c)(1 + K_d / [\text{H}^+])}$$
(1)

$$k_{cat} = \frac{\frac{k_{cat}^{int2}}{1 + K_{c,s}/[H^+])} + \frac{k_{cat}^{int}}{1 + [H^+]/K_{c,s}}}{(1 + [H^+]/K_{a,s})(1 + K_{b,s}/[H^+])}$$
(2)

$$K_{\rm p} = \frac{K_{\rm p}^{\rm int} (1 + K_{\rm a}/[{\rm H}^+])(1 + K_{\rm b}/[{\rm H}^+])(1 + [{\rm H}^+]/K_{\rm p})}{(1 + K_{\rm a,p}/[{\rm H}^+])(1 + K_{\rm b,p}/[{\rm H}^+])(1 + [{\rm H}^+]/K_{\rm p,p})}$$
(3)

where [H+] is the hydrogen ion concentration;  $k_{cat}/K_m^{int}$ ,  $k_{cat}^{int}$ ,  $k_{cat}^{int2}$ , and  $K_p^{int}$  are pHindependent constants;  $K_a$ ,  $K_b$ ,  $K_c$ , and  $K_d$  are the acid dissociation constants of uncomplexed enzyme;  $K_p$  is the acid dissociation constant of uncomplexed inhibitor; and those constants with a subscript "s" or "p" are for the enzyme\*substrate or enzyme\*product complexes. In equation 1, the terms associated with the dissociation constants  $K_c$  and  $K_d$  were not included in the fit when there was no indication of their presence. As no indication for the  $pK_a$  of  $K_{p,p}$  was present, it was set arbitrarily at 2.0 (see the discussion). The value used for  $pK_p$  was 5.8 (Tanokura, 1983). The free enzyme values for  $K_p$  were obtained from the  $k_{cat}/K_m$  profiles. The calculated values for the  $pK_a$ 's and intrinsic parameters are shown in Table 2.3.

The use of the integrated rate equation was found to give slightly higher values of  $k_{cat}$  (by 17%) and  $k_{cat}/K_m$  (by 8%), and better fits as measured by lower errors in the parameters of ~ 25% (data not shown).

At a concentration of 100 mM and a pH of 5.53, MES buffer competitively inhibited substrate and product binding by a factor of 2.1-fold. The inhibition was only 1.18-fold at pH 6.03, and was not apparent at pH 6.56. This result argues for involvement in binding of at least one of the active site histidines in its protonated state.

#### DISCUSSION

#### Brief Review of the mechanism and pH-dependence of RNase A.

RNase A specifically catalyzes the two-step hydrolysis of the  $P-O_5'$  bond of RNA after pyrimidine residues. Figure 1.1 depicts the consensus mechanism for these reactions. In the transphosphorylation step, His12 functions as a general base and His119 as a general acid. The slow hydrolysis of the 2',3'-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation. The pH-rate profiles for RNase A are generally interpreted in terms of the titration of these two histidine residues.

pH-rate profiles of  $k_{cat}/K_m$  and  $k_{cat}$ , then, would be expected to be bell-shaped, with slopes approaching unity on either side when plotted in log-log format. The  $k_{cat}/K_m$  profiles that have been reported are indeed bell-shaped, while the  $k_{cat}$  profiles vary with the substrate (Herries *et al.*, 1962; delRosario & Hammes, 1969; Machuga & Klapper, 1977; Eftink & Biltonen, 1983). Workers using cCMP as the substrate have found a slope of one for the  $k_{cat}$  profiles; those using cUMP have found a slope of less then one. Slope here refers to the acidic leg of the  $k_{cat}$  profile. The large  $K_m$ 's and strong product inhibition observed for the hydrolysis step at high pH makes the basic leg difficult to measure. These factors are likely the cause of the discrepant values reported for  $k_{cat}$  at high pH (Herries *et al.*, 1962).

The  $k_{cat}$  profile using cCMP as substrate has been modeled in terms of the two active-site histidines residues (Herries *et al.*, 1962; Eftink & Biltonen, 1983), while the  $k_{cat}$  profile using cUMP as substrate has been ascribed to a parallel pathway (delRosario & Hammes, 1969). For the latter to be correct, the alternate pathway must affect  $K_m$ , as well as  $k_{cat}$ , to such an extent that no inflection is observed in the  $k_{cat}/K_m$  profiles.

Our data for  $k_{cat}/K_m$  for the substrate cUMP agree with that of previous studies, with the exception of a second low pH limb near pH 4. Our  $k_{cat}$  data is most similar to that of Eftink & Biltonen (1983)—who also attempted to account for product inhibition through the use of the integrated rate equation—with a slope approaching one at values of pH less than ~4.5 and higher values of  $k_{cat}$  and  $K_m$  at values of pH >7 than reported by others (delRosario & Hammes, 1969; Machuga & Klapper, 1977).

#### The differential activity of the enzymes on various substrates

Two conclusions can be drawn from the data in Table 2.1. First, on a percent basis, the variation of  $k_{cat}$  for the three proteins versus the three substrates is quite small. In other words, the mutant enzymes are roughly equally handicapped for the transphosphorylation of a dinucleotide, and of a polymeric substrate, and for the hydrolysis of a cyclic nucleotide. The ability of His119 to occupy two sites through rotation about the C<sub> $\alpha$ </sub>-C<sub> $\beta$ </sub> bond—one hydrogen bonded to Asp121 (position A) and one not (position B)—and the apparent existence of a favorable orientation for catalysis in both positions suggest that this

conformational flexibility might be designed to allow for transphosphorylation to occur in one position and hydrolysis in another (Borkakoti *et al.*, 1982; Rico *et al.*, 1991) (Figure 4.8). As our mutants would be expected to preferentially affect position A over position B, a divergence in rate reduction would be expected for the two steps were this to be the case.

We have argued that in the D121N mutant, the protonated state of His119 would not be able to form a hydrogen bond to Asn121 (see Chapter 4). Crystallographic studies of a D121N semisynthetic RNase A (deMel *et al.*, 1992) and of D102N trypsin (where Asp102 is part of the catalytic triad) (Sprang *et al.*, 1987) support this argument. If position A *were* the active conformation, then one would expect a larger reduction in rate than that observed for D121N RNase A. The results of our analysis of the stability of wild-type, D121N, and D121A RNase A suggest that position A is dominant in the wild-type protein (see Chapter 4). If this is the case, one would expect D121N RNase A to be more active than wild-type RNase A. The fact that neither scenario prevails argues that both positions have similar activity.

Secondly, the presence of a purine base in what is known as the B2 site (the site 3' to the scissile bond) appears to inhibit substrate binding in the mutants. That is, the mutants'  $K_m$ 's for UpA are more perturbed versus wild type than they are for the other substrates. Crystallographic studies show that in position B, His119 appears to be in position to inhibit binding of a nucleotide. The larger size of purine bases versus that of pyrimidine bases would likely result in unfavorable van der Waals interactions between purine bases and His119 when His119 is in position B. The mutants, as noted above, are likely to shift the A:B equilibrium in favor of position B because of the loss of the His\*\*\*Asp interaction.

#### Dissection of pH dependence

Some, but not all, of our pH profiles are not consistent with a model in which only the two active-site histidines affect catalysis. The  $k_{cat}/K_m$  profiles can be fit quite well with this

simple model—aside from a second low pH limb and, for D121N, a second high pH limb. The  $K_p$  profiles also fit this model well with allowance made for the perturbation of the  $pK_a$  of the phosphate group of 3'-UMP. The  $k_{cat}$  profiles, however, show obvious deviations from the titration behavior expected of this model with slopes of less than unity observed between pH ~4.5 and 7.

We have explored a number of possibilities in attempting to explain the profiles. These possibilities include a change in rate-limiting step, kinetic versus thermodynamic  $pK_a$ 's, alternative pathways (perhaps from multiple reactive protonic states), and overlapping kinetically significant ionizations (Brocklehurst, 1994).

A change in rate-limiting step with pH for the hydrolysis reaction appears unlikely. The value of  $k_{cat}$  is slow at 10 s<sup>-1</sup> for wild-type RNase A and 0.5 s<sup>-1</sup> for the D121A mutant. If the enzyme were reverse protonated, one might argue that the intrinsic  $k_{cat}$  is high (at least for wild type), that the formation of the productive protonation state is slow, and the partition between the enzyme\*substrate complex in its productive protonation state between proceeding through the chemical step or going back to the reverse protonated state is roughly equal with the reverse step being dependent on pH. Such an argument would require that at some pH the rate-limiting step is simply the barrier to reaching the proper protonation state due to an equal or lesser amount of reverse protonation (as the p $K_a$  of His119 might well be lower, as indeed it is, see Table 2.3 and Chapter 3) and a greater barrier in the chemical step. Thus, the effect of a change in the rate-limiting step would be most apparent for wild type and possibly not at all apparent for the mutants. Because all of the enzymes display a similar slope of less than one at neutral to acidic pH, we believe that the rate-limiting step has not changed.

Relaxation studies found no relaxation step slower than the chemical step and thus RNase A is not "sticky" (Erman & Hammes, 1966). In addition, the off rate for the enzyme-substrate complex ( $\sim 10^4$  s<sup>-1</sup>) is much faster than  $k_{cat}$  and thus the pKa's are not

kinetic. Perhaps the most convincing evidence are the trapping experiments by this laboratory (Thompson *et al.*, 1994) which show that upon transphosphorylation of UpA, cUMP is released from the enzyme approximately 1000-fold faster than it gets hydrolyzed. The germane point here is that after transphosphorylation, RNase A is believed to be in the proper protonation state to carry on the second step but the partition greatly favors the release of the substrate rather than catalysis.

The pH dependence of  $K_m$  and  $K_p$  also indicate that, at least to a first approximation, RNase A is not sticky. These profiles are similar with the exception that  $K_p$  rises much more quickly as the pH falls, as is to be expected if the dianionic form of the product binds more tightly then the monoanionic. As  $K_p$  is an equilibrium binding constant,  $K_m$  would also appear to be so.

We are left to conclude that the explanation for our  $k_{cat}$  data lies in the presence of an alternative pathway produced by the titration of a residue other than the two active-site histidines. The  $k_{cat}$  data for all three enzymes fit a model with an alternative pathway well, with the p $K_a$  of the group responsible being ~5.0. No justification could be found for fitting  $k_{cat}/K_m$  with the same. Hence, the effect of the additional pathway appears to be offset by changes in  $K_m$ . It was not found necessary to fit  $K_p$  with such a group, although a detailed NMR analysis indicates that such a  $pK_a$  does affect the chemical shift of the active-site histidines when in the presence of inhibitor (see Chapter 3).

So what is this group that is responsible for this  $pK_a$ ? The necessity for offsetting changes in  $K_m$  argues that the  $pK_a$  of this group must be greater in the free enzyme than that of the complexed enzyme. This fact seemingly precludes the participation of any aspartate or glutamate residue as no such highly perturbed  $pK_a$ 's have been observed (Santoro *et al.*, 1979). The most logical residues for such an assignment—the histidines—have welldocumented  $pK_a$ 's both in the presence and absence of inhibitors (see Chapter 3 and references within). Thus, we appear to be left without a residue to which we could reasonably assign this  $pK_a$ .

#### pH behavior of kcat/Km

The  $k_{cat}/K_m$  profile for wild-type RNase A can be fit quite well with the assumption of the participation of His12, His119, and a third ionizable group with a  $pK_a$  of 3.8. Fitting of the D121N enzyme required an additional high pH  $pK_a$ . The profile of the D121A enzyme did not did not reach the extremes of pH necessary to see more than the effect of the two histidines. The  $pK_a$ 's for the two histidine residues of wild-type RNase A (Table 2.3) agree reasonably well with the NMR results of 5.8 for His12 and 6.2 for His119 (see Chapter 3 and (Meadows *et al.*, 1969; Roberts *et al.*, 1969; Markley, 1975; Patel *et al.*, 1975)). The differences are within error of any given  $pK_a$ , but recall that in pH versus rate profiles, when the two  $pK_a$ 's have similar values, the average is much better determined then the individual values. This average is 0.2  $pK_a$  units lower in this study than that observed in the NMR studies and suggests that a deuterium isotope effect may be present (D<sub>2</sub>O is the solvent in the NMR experiments). The  $pK_a$ 's for the D121N and D121A enzymes are similar to those of wild-type RNase A, as they were found to be our NMR analysis (see Chapter 3).

### High pH behavior of kcat/Km of D121N

The  $k_{cat}/K_m$  profile of D121N RNase A has a dip at high pH that is absent from the profile of the wild-type enzyme. This dip was fit with a  $pK_a$  of 9.1. This dip could possibly result from Lys41, a residue known to contribute  $10^5$ -fold to catalysis: almost completely through  $k_{cat}$ (Messmore *et al.*, 1995). The calculated  $pK_a$  is in agreement with NMR measurements of Lys41's  $pK_a$  as being 8.8 to 9.0 (Jentoft *et al.*, 1981; Blackburn & Moore, 1982). Why this  $pK_a$  does not show up in the profile of the wild-type enzyme (not simply in this study, but historically) is quite an enigma. Possibly the role of Lys41 is simply that of a hydrogen bond
donor. However, given its large contribution to catalysis and its proximity to the negatively charged phosphate group, a significant reduction in rate would seem likely upon its deprotonation. The small effect on catalysis suggests the presence of an offsetting event, such as specific base catalysis. If this offsetting event is less effective in the mutant, for whatever reason, then the  $pK_a$  of Lys41 might become apparent.

# Low pH behavior of $k_{cat}/K_m$

Our data show a  $pK_a$  of 3.8 for the  $k_{cat}/K_m$  profiles of wild-type and D121N RNase A. This  $pK_a$  is not seen in the  $k_{cat}$  profiles, indicating that this group is involved in binding of the substrate. Eftink and Biltonen (1983) reported no such inflection for cUMP but they did see one with cCMP as the substrate which they attributed to the pyrimidine base of cCMP. However, the latter was not observed by Rabin and coworkers for the same substrate (Herries *et al.*, 1962). cUMP has no  $pK_a$  in this neighborhood. Therefore, it would appear that a group on the enzyme is responsible and that Asp121, a likely candidate, is not the group in question.

The fact that the  $K_p$  profiles do not show the same  $pK_a$  can be explained by either a differential mode of binding or by the presence of an overlapping ionization. The second  $pK_a$  of the phosphate of bound 3'-UMP has been measured by NMR to be 4, suggesting that the latter is the correct explanation (see Chapter 3 and (Gorenstein & Wyrwicz, 1973; Haar *et al.*, 1973; Tanokura, 1983)). Furthermore, the binding constants at acidic pH for the substrate and product (with a largely monoanionic phosphate) are similar suggesting a similar mode of binding.

Karpeisky and Yakovlev (1981) have argued, based upon NMR titrations with and without inhibitors and upon X-ray diffraction analyses, that Asp83 forms a hydrogen bond with the sidechain of Thr45. Protonation of Asp83 presumably disrupts this hydrogen bond and allows Thr45 to assume an alternative conformation in which its hydroxyl group experiences van der Waals repulsion from the N3H proton of the base. We propose that both cUMP and 3'-UMP do not bind as well to this conformation as to the other.

# pH behavior of kcat

Numerous NMR studies have shown that His119 is much more perturbed than is His12 upon binding 3'-UMP. By analogy, a similar pattern is expected for the binding of cUMP (see Chapter 3 and references within). Given the postulated roles of His119 as a base and His12 as an acid in the hydrolysis reaction, the wild-type enzyme must be reverse protonated, that is it proceeds via a minor ionization state. This reverse protonation explains, in part (Thompson *et al.*, 1994), why hydrolysis is so much slower than transphosphorylation.

We shall assume that the  $pK_a$  of His119 in the  $k_{cat}$  profiles is that of the higher value. This value is consistent with an NMR titration of these enzymes in the presence of 3'-UMP (Chapter 3). The  $pK_a$ 's for His119 determined from the NMR study for the complex of 3'-UMP with wild-type, D121N, and D121A RNase A were reported to be 7.9, 7.5, and 6.9, respectively. For His12, these values were reported to be 6.5, 6.7, and 6.8, respectively. These values agree well with the ones reported in Table 2.3 for D121N and D121A RNase A, and for His12 of wild type. However, for His119 of the wild-type protein there is a discrepancy of 0.8  $pK_a$  units, with the  $pK_a$  determined in this study being the larger of the two. This discrepancy may reflect a failure to achieve sufficient saturation in the NMR study. It may also in part reflect the errors in  $k_{cat}$  brought about through failure to obtain sufficient saturation. Alternatively, perhaps the interactions of cUMP and 3'-UMP with His119 in the wild-type enzyme are different.

# Behavior of the K<sub>p</sub> profiles

D121A RNase A binds twofold more tightly to 3'-UMP than does D121N RNase A, and it has a lower  $k_{cat}$ . These results are consistent with the mutation to alanine producing an unproductive (or less productive) binding mode. NMR experiments indicate that for the D121A enzyme, the perturbation of His119 is significantly smaller and that of His12 significantly larger in the presence of 3'-UMP than are the analogous perturbations of the other two enzymes (Chapter 3). Summing up the perturbations and dividing by two to generate an "average"  $pK_a$ , one curiously finds that for D121A RNase A, the perturbation is slightly smaller (by 0.27 pK<sub>a</sub> units) than that for the other two (which are within 0.08 pK<sub>a</sub> units). This difference by itself argues for a decreased affinity of D121A RNase A for 3'-UMP. There must therefore exist elsewhere an increased interaction, which again argues for a binding mode in the D121A enzyme that is somewhat different from that in the other two proteins. The  $pK_a$ 's determined from the  $K_p$  profiles do not appear to agree with our NMR. results (Chapter 3). This discrepancy is possibly due to the errors found in the  $K_p$  profiles. The tight correlations for many of the parameters in these profiles generates greater uncertainty in the data. The errors reported do not take into account the errors in the  $pK_a$ 's of the free enzyme because, as noted above, these were fixed at the values found for the  $k_{cat}/K_{m}$ profiles.

# Comparison of $K_m$ and $K_p$

At low pH both substrate (cUMP) and product (3'-UMP) have a similar affinity for the enzyme but at high pH the product binds approximately 20-fold more tightly. This dichotomy suggests a role for the second ionization of 3'-UMP in binding. The similarities observed for the  $pK_a$ 's of the active-site histidines in the enzyme substrate complex versus that observed in the study of the NMR titration of the enzyme product complex suggest the involvement of a residue other than the active-site histidines. The active-site residue Lys41 is a possible candidate in this increased interaction as NMR studies indicate that its  $pK_a$  is perturbed by

0.3 upon binding 3'-UMP (Jentoft *et al.*, 1981). However, this value is not sufficient in and of itself to explain all the observed difference, because mutants that lack a positive charge at position 41 only suffer a 4-fold increase in  $K_m$  (Messmore *et al.*, 1995). Given the lack of any other positively charged group in the immediate vicinity, the increased stability would appear to be due to either "non-specific" long-range interactions with the multitude of basic residues contained in this basic protein or to a charge – dipole interaction, possibly with Gln11, a residue that has been shown by X-ray diffraction analysis to form a hydrogen bond to a non-bridging oxygen of the phosphoryl group (Wlodawer, 1985), and whose role has been postulated to be that of preventing non-productive binding (delCardayre *et al.*, 1995).

#### Lags

Lags in which a couple of minutes were needed before the rate of substrate turnover reached its maximum were observed in numerous instances for cUMP, sporadically for poly(C) and not at all for UpA. The lags were independent of the addition sequence of substrate and enzyme, and were not an artifact of thermal equilibration. Addition of a second aliquot of enzyme after the initial velocity had been obtained resulted in a second lag. That is, the velocity did not increase immediately in response to the second aliquot. In general the lags appeared much more pronounced at substrate concentrations above the  $K_m$ , but at the extremes of pH (3.45, 8.46, 9.00) they occurred at all substrate concentrations. The lag was observed to be inversely proportional to the enzyme concentration for any given protein but not to be dependent on its absolute concentration, as lags of similar duration were observed when the three proteins had similar initial velocities. Being of different activity, the protein concentrations necessary to produce matching initial velocities were, of course, different. Eftink & Biltonen (1983) also reported lags for the turnover of cUMP at pH 4 and below. Perhaps the use of higher concentrations of enzyme and/or lower concentrations of substrate

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precluded the observation of the lags in other studies (or perhaps they were treated as artifacts).

An explanation for these lags is difficult to come by. One possibility is the presence of a tightly binding but slowly hydrolyzing molecule. This explanation is precluded, however, by the second aliquot results. The observation that the lag was inversely proportional to the enzyme concentration is consistent with the formation of a dimeric enzyme because the half life for such an event is first order in enzyme concentration, but it is inconsistent with the lag being dependent of absolute enzyme to bind more than one substrate molecule. At the present time, we must concur with Eftink & Biltonen (1983) and refer to the lags as inexplicable.

# Noncovalent Disaggregation of cUMP

Upon dilution of thermal equilibrated cUMP into the reaction buffer to a final concentration of 50 mM the absorbance was observed to decrease in an exponential fashion over the course of approximately 4 min. Addition of enzyme either before or immediately after the addition of cUMP removed the absorbance change. The obvious conclusion is that RNase A is catalyzing the noncovalent depolymerization of cUMP. Given that RNase A is designed to bind polymeric RNA, this finding should not be so surprising. We see no direct way to relate this observation to the lags observed in the initial velocities.

# Role of Asp121

In spite of the proximity of Asp121 to the residue implicated as being most important to binding—His119—its own role in binding is minimal at best. Our mutants at position 121 largely affect  $k_{cat}$  and not  $K_m$  or  $K_p$ . The observation that wild-type and D121N RNase A differ by only 1.8-fold in rate at pH 3.5 argues strongly against a charge relay mechanism in which Asp121 removes a proton from His119, which in turn removes one from water to

facilitate attack on the cyclic phosphodiester. Indeed, such a small decrease in rate argues against any significant role for Asp121 in catalysis and suggests that the role of Asp121 is to be found elsewhere.

substrate	enzyme	$k_{\text{cat}}$ (s <sup>-1</sup> )	% a	<i>K</i> <sub>m</sub> (mM)	% <sup>b</sup>	k <sub>cat</sub> /K <sub>m</sub>	% C
cUMP	wild-type	3.7±0.2	100	2.3±0.5	100	1.6±0.3	100
	D121N	1.55±0.11	42	2.7±0.7	85	0.58±0.1	36
	D121A	0.187±0.006	5.1	1.6±0.2	140	0.11±0.01	7
UpA	wild-type	890±30	100	0.38±0.03	100	2300±190	100
	D121N	190±20	21	1.6±0.2	25	120±20	5
	D121A	30±3	3.4	2.2±0.3	17	14 <del>±</del> 2	0.6
poly(C)	wild-type	475±10	100	0.14±0.01	100	3300±200	100
	D121N	51±2	11	0.10±0.01	140	510 <del>±6</del> 0	15
	D121A	8.2±0.3	1.7	0.20±0.02	70	40±4	1.2

# TABLE 2.1 Steady-state kinetic parameters for catalysisby wild-type, D121N, and D121A RNase A at pH 6.0

<sup>a</sup> Defined as  $k_{cat}^{mutant/k_{cat}}$  wild-type. <sup>b</sup> Defined as  $(k_{cat}/K_m)^{mutant/(k_{cat}/K_m)}$  wild-type. <sup>c</sup> Defined as  $K_m^{wild-type/K_m}$  mutant

	wild-type			D121N			D121A				
pH <sup>a</sup>	$k_{\rm cat}$ (s <sup>-1</sup> )	K <sub>m</sub> (mM)	<i>K</i> <sub>p</sub> (mM)	$k_{\rm cat}$ (s <sup>-1</sup> )	K <sub>m</sub> (mM)	<i>K</i> <sub>p</sub> (mM)	kcat (s <sup>-1</sup> )	<i>K</i> <sub>m</sub> (mM)	<i>K</i> p (mM)	Δε <sub>282</sub> b	$\Delta \epsilon_{293} b$
3.45 (F)	.042±.001	3.79±0.2	2.7	.029±.001	5.8±0.6	3.4				958	44.3
4.00 (F)	.156±.003	2.16±0.07	0.79	.096±.003	3.1±0.2	0.68				995	49.1
4.48 (A)	0.43±0.01	1.45±0.10	0.26	.216±.006	2.3±0.2	0.28	.031±.001	1.66±0.06	0.17	971	47.7
5.02 (A)	1.05±0.01	1.34±0.03	0.13	0.47±0.02	1.6±0.2	0.10	.055±.002	.957±.007	0.072	1000	
5.53 (M)	1.9±0.1	2.1±0.2	0.22	0.68±0.02	1.8±0.1	0.17	.090±.002	1.17±0.04	0.085	1050	
5.58 (A)	1.96±0.04	1.04±0.06	0.090	0.73±0.04	0.9±0.1	0.070				1050	
6.00 (T)	3.7±0.2	2.2±0.3	0.160							1140	53.8
6.03 (M)	3.7±0.2	2.7±0.5	0.16	1.55±0.11	3.2±0.4	0.20	.187±.006	1.9±0.3	0.071	1140	53.8
6.48 (B)	5.5±0.2	4.6±0.4	0.23							1180	55.0
6.56 (M)	5.9±0.2	5.5±0.6	0.26	2.71±0.14	7.9±1.2	0.60	.324±.004	5.0±0.2	0.145	1180	55.0
6.94 (P)	9.2±0.4	21±3	0.84	2.97±0.12	17±2	1.05	0.43±0.02	15±1.5	0.38	1240	57.3
7.02 (T)	8.9±0.8	21±5	0.79							1240	57.3
7.46 (P)	9.5±0.8	63±11	2.20	1.46±0.08	26±4	1.6	.169±.006	17±2	0.76		58.7
7.97 (B)	7.5±0.7	160±20	7.10	0.73±0.04	50±7	3.3					59.2
8.46 (B)	6.4±0.7	390±50	12.5	0.42±0.02	110±10	5.2					57.7
9.00 (S)	0.0050 c		<u>19.0</u>	0.0010 C		5.0					57.0

TABLE 2.2 pH-Dependence of the steady-state kinetic parameters for the hydrolysis of cUMP by wild-type, D121N, and D121A RNase A

<sup>*a*</sup> Letters in parentheses denote buffer: F = formic acid, A = acetic acid, M = Mes, T = Bis-Tris, B = Bicine, P = Mops, S = Ampso.<sup>*b*</sup> In M<sup>-1</sup>cm<sup>-1</sup>. <sup>*c*</sup> Represents  $k_{cat}/K_m$  rather than  $k_{cat}$ .

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parameter	enzyme	pKa	р <i>К</i> ъ	pK <sub>c</sub>	p <i>K</i> d	$k_{\text{cat}}/K_{\text{m}}^{\text{int}}$ (mM <sup>-1</sup> s <sup>-1</sup> )	
k <sub>cal</sub> /K <sub>m</sub>	wild-type	5.58±0.20	6.01±0.17	3.80±0.12		4.86:	±1,76
	D121N	5.48±0.13	6.01±0.06	3.80±0.17	9.09±0.17	1.60:	±0.27
	D121A	5.68±0.97	5.84±0.96			0.45±0.95	
		5.47±0.08ª	6.07±0.12				
		pK <sub>a,s</sub>	pK <sub>b,s</sub>	pK <sub>c,s</sub>		kcat <sup>int1</sup> (s <sup>-1</sup> )	kcat <sup>int2</sup> (s <sup>-1</sup> )
k <sub>cat</sub>	wild-type	6.47±0.14	8.63±0.16	4.99±0.15		10.5±1.0	<b>49±</b> 14
	D121N	6.32±0.28	7.44±0.15	4,52±0.29		3.9±1.1	24±15
	D121A	6.66±0.22	6.95±0.21	4.62±0.75		1.0±0.3	6.5±3.2
		р <i>К</i> а,р	pK <sub>b,p</sub>			Kp <sup>int</sup> (mM <sup>-1</sup> )	
Kp	wild-type	6.12±0.10	8.53±0.05			0.023±0.002	
	D121N	6.03±0.12	7.60±0.10			0.020	±0.002
	D121A	6.00±0.09	7.83±0.04			0.013	±0.001

TABLE 2.3 Apparent  $pK_a$ 's and intrinsic parameters for the hydrolysis of cUMP by wild-type, D121N, and D121A RNase A

<sup>a</sup>Here the  $pK_a$ 's for D121A were constrained to be 0.6  $pK_a$  units apart as has been suggest by Cleland (1977) when two  $pK_a$ 's have similar values. The fit in Figure 2.1 uses the first values listed.

Figure 2.1 Graph of  $log(k_{cat}/K_m)$  versus pH for wild-type (O), D121N (X), and D121A RNase A (+). The lines represent the nonlinear least-squares fits of the data as described in the text. The values for  $k_{cat}/K_m$  of D121N RNase A were multiplied by a scaling factor of 2.2. The values for  $k_{cat}/K_m$  of D121A RNase A were multiplied by a scaling factor of 13.



Figure 2.2 Graph of  $log(k_{cat})$  versus pH for wild-type (O), D121N (X), and D121A RNase A (+). The lines represent the nonlinear least-squares fits of the data as described in the text. The values for  $k_{cat}$  of D121N RNase A were multiplied by a scaling factor of 1.6. The values for  $k_{cat}$  of D121A RNase A were multiplied by a scaling factor of 16.



Figure 2.3 Graph of  $-\log(K_p)$  versus pH for wild-type (O), D121N (X), and D121A RNase A (+). The lines represent the nonlinear least-squares fits of the data as described in the text.



# Chapter 3

His---Asp Catalytic Dyad of Ribonuclease A:  $pK_a$ 's of the Histidine Residues in the Wild-Type, D121N, and D121A Enzymes ABSTRACT Bovine pancreatic ribonuclease A (RNase A) has a catalytic dyad in its active site. Structural analyses had indicated that Asp121 forms a hydrogen bond with His119, which is a general acid catalyst during RNA cleavage. Here, the <sup>1</sup>H-NMR spectra at 400 MHz of wild-type, D121N, and D121A RNase A were analyzed as a function of pH. The  $pK_a$  of His119 dropped by 0.10, while that of His12 rose by ~ 0.15. A combination of the known conformational mobility of His119 and a postulated rearrangement of the enzyme is concluded to be responsible for the observed  $\Delta pK_a$ 's. The  $pK_a$ 's of the active-site histidine residues in the presence of the inhibitor, uridine 3'-phosphate (3'-UMP), revealed a pattern in which a reduction in the perturbation of His119 was offset by a similar increase in perturbation of His12. Comparison of the titration curves of the free enzyme with that obtained in the presence of two concentrations of inhibitor. At the lower concentration of inhibitor, an apparent  $pK_a$  of 5.2 was observed in the titrations of His119 and His12. Such a  $pK_a$  was not observed at the higher concentration of inhibitor, perhaps as a result of the second molecule freezing out an associated conformational change.

# INTRODUCTION

Bovine pancreatic ribonuclease A (RNase A EC 3.1.27.5) has been the object of much landmark work in biophysics and biochemistry (Richards & Wyckoff, 1971; Karpeisky & Yakovlev, 1981; Blackburn & Moore, 1982; Wlodawer, 1985; Eftink & Biltonen, 1987). For example, RNase A was the first enzyme and second protein (after insulin) for which a complete amino acid sequence was determined, and the third enzyme and fourth protein (after myoglobin, lysozyme, and carboxypeptidase) whose structure was solved by X-ray diffraction analysis. Also, the use of NMR spectroscopy in elaborating both protein structure (Saunders *et al.*, 1957) and protein folding pathways (Udgaonkar & Baldwin, 1988) was developed with RNase A. The ensuing wealth of information has made RNase A an ideal model system for detailed biophysical analyses.

RNase A is a small protein (124 amino acid residues; 13.7 kDa) that catalyzes the hydrolysis of RNA in two distinct steps (Figure 1.1). In the first step, the sidechain of His12 acts as a general base that abstracts a proton from the 2'-hydroxyl of a substrate molecule and thereby facilitates attack on the phosphorus atom. The sidechain of His119 acts as a general acid that protonates the 5'-oxygen to facilitate its displacement. Both products are then released to solvent. The slow hydrolysis of the 2',3'-cyclic phosphodiester occurs in a separate step that resembles the reverse of transphosphorylation (Thompson *et al.*, 1994).

RNase A may have converged upon a catalytic dyad that is similar to the catalytic triad of lipases and serine proteases. Neutron diffraction analysis indicates that Asp121 forms a hydrogen bond with His119 (Wlodawer & Sjölin, 1981). Specifically, Asp121 may stabilize the tautomer of His119 that is appropriate for both the delivery of a proton to the leaving nucleoside and the abstraction of a proton from the water molecule that attacks the cyclic phosphodiester intermediate (Wlodawer & Sjölin, 1981). Alternatively, complete proton transfer between Asp121 and His119 may occur (Umeyama *et al.*, 1979). The importance of

Asp121 to catalysis by RNase A is also evident from Asp121 being conserved in homologous ribonucleases from 41 distinct species (Beintema, 1987).

Here, we have used NMR spectroscopy to gain insight into the role of Asp121 during catalysis. Specifically, we have determined how the  $pK_a$  of His119 is changed if Asp121 is replaced with an asparagine or alanine residue. We extend this analysis to the  $pK_a$  of His12 and the two other histidine residues in RNase A. Finally, we determined the effect of a subsaturating and a saturating concentration of the hydrolysis product uridine 3'-phosphate (3'-UMP) on the histidine  $pK_a$ 's in all three enzymes. This analysis enabled us to determine  $K_d$  for the enzyme\*product complex as a function of pH. We were surprised to learn of the existence of both a second binding site for 3'-UMP and an additional enzymic  $pK_a$ , which were revealed only through a global analysis of the data.

# MATERIALS AND METHODS

#### Materials

RNase A (Sigma Type XII-A, lyophilized, salt free) was exhaustively dialyzed versus H<sub>2</sub>O. 3'-UMP (Sigma, 98%) was used without further purification. D121N and D121A RNase A were produced in *Escherichia coli* and purified as described in Chapter 2. D<sub>2</sub>O (99.9%), DCl (35% v/v in D<sub>2</sub>O), and NaOD (40% v/v in D<sub>2</sub>O) were from Isotec (Miamisburg, OH). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was from Cambridge Isotope Laboratories (Andover, MA).

# Procedures

Both the deuterium exchange procedure and the NMR titrations were performed at a protein concentration of 50 mg/mL. pH\* represents a direct reading of pH, not corrected for the deuterium isotope effect. Concentration of RNase A was determined by using an absorption

coefficient of  $\varepsilon_{1 \text{ cm}}^{0.1\%} = 0.72$  at 277.5 nm (Sela *et al.*, 1957). Concentration of 3'-UMP was determined by mass.

To perform pH titrations of protein, exchangeable hydrogen atoms must be replaced with deuterium atoms. To exchange hydrogen for deuterium, lyophilized protein was dissolved in D<sub>2</sub>O, lyophilized again, and redissolved in D<sub>2</sub>O. The pH\* was adjusted to 3.0 with DCl, and the resulting solution was heated to 60 °C for 1 h to exchange the amide protons (Markley, 1975). NaCl and DSS were then added to final concentrations of 0.2 M and 0.5 mM, respectively. 3'-UMP was added from a stock solution of 270 mM.

Proton NMR spectra were recorded on a Bruker DMX 400 MHz spectrometer at 25 °C using 16K data points and an acquisition time of 1.5 s with 128, 256, or 512 scans. Chemical shifts are reported with respect to DSS. pH\* was adjusted using 1/10 dilutions of DCl and NaOD. All data were fit using nonlinear least squares.

# RESULTS

The  $pK_a$ 's of His12, His119 and His105 in wild-type, D121N, and D121A RNase A were well determined. The largest contribution to error appears to lie in the measurement of pH itself. Such systematic errors largely disappear when differences in  $pK_a$ 's are calculated in any given titration.

# Titration of the C(2)-H peaks of unliganded proteins

Results of the NMR titration of the unliganded proteins are shown in Figure 3.1, and the calculated  $pK_a$ 's and limiting chemical shifts are presented in Table 3.1. The apparent interaction between the two active-site histidine residues—His12 and His119—necessitated a fit involving microscopic  $pK_a$ 's for these two residues. The microscopic  $pK_a$ 's indicated that protonation of the active-site histidine residues had negative cooperatively—protonation of

one histidine residue disfavored the protonation of the other, as had been observed previously (Markley & Finkenstadt, 1975). The cooperatively was slightly smaller in the previous study than that observed here. The previous observations were made in the presence of a higher ionic strength (0.30 M versus 0.20 M NaCl), which would be expected to reduce Coulombic interactions.

The mutations of Asp121 decreased the  $pK_a$  of His119 by 0.11 (averaged microscopic values), and increased the  $pK_a$  of His12 by approximately 0.15. In all three proteins, the titration of His12 displayed an inflection in the acidic region. The proximity of this inflection to the  $pK_a$  of His12 and the lack of data below  $pH^*$  3 made for a large error in its determination. In all three proteins, the titration of His48 also displayed an inflection in the acidic region, as had been seen previously (Markley, 1975). The effect of the mutations on the titration of His48 was minor. No effect was observed on the titration of His105. The limiting chemical shifts of each histidine residue were almost identical for all three proteins.

# Titration of the C(2)-H peaks of protein-inhibitor complexes

NMR titrations of wild-type, D121N, and D121A RNase A were performed in the presence of the reaction product 3'-UMP. In these titrations, the concentration of protein was 3.5 mM and that of 3'-UMP was either 1.5 mM or 11 mM. The results are shown in Figures 3.2 and 3.3, and the calculated  $pK_a$ 's and limiting chemical shifts are presented in Table 3.1. The data were fit without regard to the disassociation of inhibitor.

In the wild-type enzyme, His119 was more perturbed by the presence of 3'-UMP than was His12, as had been observed previously (Eftink & Biltonen, 1987). In the presence of 11 mM 3'-UMP, mutation of Asp121 to an asparagine residue reduced the perturbation of the  $pK_a$  of His119 by 0.36 versus wild type and increased that of His12 by 0.21. Mutation of Asp121 to an alanine reduced the perturbation of the  $pK_a$  of His119 by 0.36. Comparisons are based upon the microscopic  $pK_a$ 's of His119 in

which His12 is not protonated and the microscopic  $pK_a$ 's of His12 in which His119 is protonated as this combination was the predominant one and as such the errors in their  $pK_a$ 's were small.

The presence of 3'-UMP produced a new  $pK_a$  of 3.9 for His119. For all three enzymes, protonation of His12 and His119 in the presence of 11 mM 3'-UMP showed positive rather than negative cooperatively. In other words, the microscopic  $pK_a$ 's of His12 and His119 indicated that protonation of one histidine residue favored the protonation of the other. This suggests that the phosphate is oriented more favorably with respect to its interaction with the active site histidines when both are protonated.

In the course of titrating D121N RNase A in the presence of 3'-UMP, we observed that the C<sub>4</sub> proton peak of His119 was shifted downfield at acidic pH by ~0.3 ppm, to a spectral region where it is was free from being obscured by the signals of phenylalanine and tyrosine residues. A comparison of spectra of D121N RNase A with that observed for wild-type enzyme led us to conclude that our assignment of the C<sub>4</sub> proton of His119 and that of Lenstra et al. (1979) are correct. Although the titration curves could only be obtained up to pH\* 6 as the peak again became obscured by the signals of phenylalanine and tyrosine residues, one point was readily apparent: the chemical shift of His119 at acidic pH in the presence 1.5 mM 3'-UMP was not between that of the free enzyme and that obtained in the presence of 11 mM 3'-UMP.

# DISCUSSION

#### Unliganded enzymes

His119 occupies two positions in wild-type RNase A (Borkakoti *et al.*, 1982). In position A, the imidazole forms a hydrogen bond with Asp121. In position B, the imidazole is removed from Asp121 by 7 Å (Figure 4.8). In both positions His119 appears able to bind substrate

and, in lieu of any evidence to the contrary, to participate in catalysis. Our observation that the  $pK_a$  of His119 drops by only 0.1 upon loss of Asp121—a value much smaller than that expected for the loss of a hydrogen bond between functional groups of opposite charge (Fersht *et al.*, 1985)—suggests that the conformational flexibility of His119 is at work here. The results of a thermodynamic study of the pH dependence of the stability of these three proteins suggests that position A is dominant in the wild-type protein (Chapter 4). How then can we explain the very small drop in the  $pK_a$  of His119 in the mutants? We propose that another residue must buffer the effect of removing Asp121 and that this residue is Glu111. This residue is only 5 Å from the imidazole group of His119 when it is in position B (Figure 4.8). Still, this explanation for the small perturbation of His119 in position A versus Glu111 and His119 in position B would argue for a  $\Delta pK_a$  larger than that observed.

Perhaps the answer lies in the curious perturbation to His12. Replacing Asp121 with an asparagine or alanine residue is shown to increase the  $pK_a$  of His12 by 0.15, an effect larger and in the opposite direction to that of His119. A simple coulombic calculation indicates that removing the negatively charged Asp121 is expected to result in a *decrease* in the  $pK_a$  of His12 by approximately 0.3. This calculation is based on the observations that protonating His12 decreases the  $pK_a$  of His119 by 0.4, and vice versa, and that the two are separated by 7 Å while the distance between His12 and Asp121 is 10 Å, and the assumption that the dielectric constant is similar for both interactions. The involvement of His119 through its conformational flexibility—in this curious perturbation of His12 is difficult to justify as its distance to His12 is very similar in both conformations and the  $\Delta pK_a$  for the microscopic  $pK_a$ 's is similar for all three enzymes. (The latter argues for a similar "effective" distance, that is, the distance with allowance made for the dielectric constant.) It would thus appear that these mutations have effects on a more global scale, quite possibly involving some of the many basic residues in RNase A. This result suggests a certain "plasticity" to the enzyme structure.

The difference between our simple calculation for the expected perturbation of His12 and that observed is 0.4  $pK_a$  units. If we assume that whatever is producing this deviation has the same effect on His119, then we can calculate the expected perturbation on the  $pK_a$  of His119 due to the removal of Asp121 to be approximately 0.5  $pK_a$  units. This seems to be a much more reasonable figure than that observed.

Our results are in conflict with those from a recent study that used a semisynthetic RNase A (Cederholm et al., 1991). This enzyme, RNase-(1-118)•(111-124), consisted of a noncovalent complex between residues 1 - 118 of RNase A and a synthetic 14-residue peptide consisting of residues 111 – 124 of RNase A. Surprisingly, replacing Asp121 of the semisynthetic enzyme with an asparagine residue increased the macroscopic  $pK_{a}$  of His119 by 0.05 and decreased the macroscopic  $pK_a$  of His12 by 0.09—results that are in opposition to ours. Most notable is the fact that the difference between the  $pK_a$ 's of His119 and His12  $(pK_a His119 - pK_a His12)$  widens to 0.45 for D121N from 0.3 for the parent semisynthetic protein, while our  $\Delta p K_a$  drops to -0.04 from 0.18 for wild type. This deviation is consistent with a difference in structure between authentic D121N RNase A and the semisynthetic D121N enzyme. Indeed, a crystallographic study on the semisynthetic mutant revealed many small changes in structure versus that of the semisynthetic wild type (deMel et al., 1992). Preliminary studies in this lab show D121N to have a structure identical to that of wild type (Schultz et al., 1995). Calculations performed by Cederholm et al. using the Poisson-Boltzmann equation (with His119 in position B) predicted a much larger perturbation for both histidines. They thus conclude, much as we do, "that the protein as a whole is accommodating (or attempting to accommodate) to the change in charge distribution resulting from the asparagine substitution".

#### Enzyme•Product Complexes

As can be seen in Table 3.1 and Figure 3.3, the mutants show a decreased perturbation of the  $pK_a$  of His119 being largely offset by an increased perturbation of His12. This result argues that the loss of interaction with one histidine is being offset by an increased interaction with another and displays, again, a certain "plasticity" to the enzyme's structure.

To extract as much as possible from the data, we fit the results for the unliganded and liganded enzyme to the thermodynamic cube shown in Figure 3.4. This analysis allows for the dissociation of the inhibitor as well as the determination of the intrinsic (that is, pH-independent) dissociation constant,  $K_d^{int}$ . To determine  $K_d$  as a function of pH\*, we used eq 1.



where [H<sup>+</sup>] is the hydrogen ion concentration,  $K_1 = K_a$  of His12 when His119 is not protonated,  $K_2 = K_a$  of His119 when His12 is not protonated,  $K_3 = K_a$  of His119 when His12 is protonated,  $K_4 = K_a$  of His12 when His119 is protonated, and  $K_p = K_a$  of the phosphoryl group of 3'-UMP. Terms containing an "T" superscript correspond to  $K_a$ 's in the

presence of 3'-UMP. To determine the percent of inhibitor bound, we inserted the values of  $K_d$  into equation 2:

$$K_{d} = \frac{(\mathbf{E}]_{\text{total}} - [\mathbf{E} \cdot \mathbf{I}])([\mathbf{I}]_{\text{total}} - [\mathbf{E} \cdot \mathbf{I}])}{[\mathbf{E} \cdot \mathbf{I}]}$$
(2)

where  $[I]_{total}$  is the total concentration of 3'-UMP,  $[E]_{total}$  is the total concentration of RNase A, and  $[E \cdot I]$  is the concentration of enzyme 3'-UMP complex. The calculated chemical shift is then simply the weighted sum of the two

$$ppm_{calc} = ppm_{free} \left( 1 - \frac{[E \cdot I]}{[E]_{total}} \right) + ppm_{bound} \frac{[E \cdot I]}{[E]_{total}}$$
(3)

Closer examination of the data, however, immediately revealed a problem with the assumption that the binding of RNase A to 3'-UMP was first-order. The observed chemical shift obtained in the presence of 1.5 mM 3'-UMP should fall between that of the free enzyme and that in the presence of 11 mM 3'-UMP. The chemical shifts of the C4 proton of His119 in D121N RNase A did not follow this pattern. Thus, the RNase A plus 3'-UMP system is a three-body one. A second molecule of RNase A would have resulted in significant line broadening, which was not observed. Instead, we conclude that a second molecule of 3'-UMP binds to the enzyme, affecting the binding of the first.

To fit the titration curves to eq 1-3, we assumed that only the active site is occupied at sub-saturating concentrations of 3'-UMP—that is, when the concentration of 3'-UMP is 1.5 mM. In this fit, the value of  $K_d^{int}$  was adjusted so as to reproduce the values of  $K_p$  that we obtained from steady-state kinetic analyses (Chapter 2). Surprisingly, we found that an additional  $pK_a$  of ~5.2 was needed to fit the titration curves. This  $pK_a$  was most obvious in the titration of His119 in wild-type and D121N RNase A. It was observed to a lesser extent

in the titrations of His119 and His12 in D121A RNase A, and in the titration of His12 in the wild-type enzyme. The titration of His12 of D121N RNase A displayed no such inflection. The chemical shift of the group associated with the  $pK_a$  of 5.2 was perturbed upfield upon protonation. It was necessary that the effect of this  $pK_a$  disappear with increasing pH with a  $pK_a$  identical or near to that of the histidine itself. No attempt was made to allow for a change in  $K_d$  with the titration of this  $pK_a$ , as the data were not sufficient to allow for such a determination. The low  $K_d$ 's observed in the range of this  $pK_a$ , however, suggest that all of the inhibitor is bound to the enzyme, so that this  $pK_a$  is not appreciably perturbed due to disassociation.

The titration curves for unliganded enzyme and for enzyme in the presence of a saturating concentration of 3'-UMP (11 mM) were also fit to equations 1-3. This fit was performed in spite of the seeming inability of the two-body model to account for the saturating inhibitor curves. Our hope was that some indication of the effect of the second site might become apparent when these results were compared to those for the 1.5 mM fit. His12 of D121N RNase A, the only active-site histidine residue that did not display the  $pK_a$  of 5.2 in the presence of sub-saturating 3'-UMP, was also the only histidine to which all three curves could be fit simultaneously. This result is consistent with our assumption that in the presence of sub-saturating 3'-UMP, only the active site is occupied, as significant binding to a second site would have resulted in a reduction in the perturbation of the active-site histidines and thus a failure to adequately fit all three titrations simultaneously. The others differed from their 1.5 mM counterparts in not displaying the  $pK_{a}$  of 5.2. They also displayed different limiting acidic chemical shifts for the bound state versus that observed at 1.5 mM 3'-UMP, with the extent to which the chemical shifts varied mirroring the size of the inflection at pH 5.2. One possible explanation for the lack of the 5.2 pK<sub>2</sub> at 11 mM 3'-UMP is that the second molecule is inhibiting a conformational change associated with this  $pK_{a}$ .

#### Acidic inflections

The inflection with a  $pK_a$  of 3.9 observed in the titration of His119 in the presence of 3'-UMP is almost certainly that of the phosphoryl group. The perturbation from 5.8 for free 3'-UMP (Tanokura, 1983) is consistent with its interaction with the two active-site histidine residues. Thermodynamic determination of  $K_p$  (Flogel & Biltonen, 1975) as a function of pH supports this view as do studies that relied on <sup>31</sup>P-NMR studies spectroscopy (Gorenstein & Wyrwicz, 1973).

The inflection seen in the titration of His12 has been widely reported, but its assignment less so. Perhaps the most thoughtful analysis of the titration behavior of His12 comes from Karpeisky and Yakovlev (1981). These workers conclude from much evidence that the group responsible is Asp83, mediated through Thr45 and its interaction with the base of the inhibitor and His12. The protonation of Asp83 is postulated to disrupt its interaction with Thr45, which then takes on an alternate conformation in which a hydrogen bond is formed between the carbonyl group and the imidazole ring of His12. The anisotropic effect of the carbonyl group, then, is responsible for the downfield shift of His12. The disappearance of this shift upon addition of 3'-UMP is attributed to the disruption of the Thr45 – His12 hydrogen bond brought about by van der Waals repulsion from the N3H proton of uridine and the hydroxyl of Thr45.

#### Plausibility of a Second Molecule

Evidence for subsites in RNase A come from many quarters. The fact that RNase A binds and cleaves polymeric substrates is suggestive in and of itself. A number of X-ray diffraction analyses on complexes with substrate analogs have revealed the presence of subsites (Eftink & Biltonen, 1987). In addition, Irie and coworkers used "P-NMR spectroscopy to provide evidence for three phosphate binding sites and two base sites (Irie *et al.*, 1984). Perhaps most germane to this work are studies that found noncovalent interactions among bases when bound to RNase A: namely that the bases adenine, adenosine, 5'-AMP, and 3'-AMP increased the enzymatic hydrolysis of cCMP (Wieker & Witzel, 1967; Haffner & Wang, 1973).

The apparent  $pK_a$  of 5.2 is not so readily explained. The  $pK_a$ 's of eight of the 11 carboxyl groups in RNase A have been determined (though few have been assigned), and none have such a high  $pK_a$  (Santoro *et al.*, 1979). The results of kinetic studies done on these proteins suggest that the  $pK_a$  of this group in the free enzyme is greater than 5.2 (Chapter 2). The only groups in RNase known to have  $pK_a$ 's in this neighborhood are the histidine residues. However, we see no evidence for such an involvement by the histidines.

There is evidence aside from that presented here for a  $pK_a$  of 5.2. Kinetic studies on the hydrolysis of cUMP reveal a slope of less than one when  $k_{cat}$  is plotted as  $log(k_{cat})$  versus pH (see Chapter 2 and (delRosario & Hammes, 1969; Eftink & Biltonen, 1983)). This result suggests that a  $pK_a$  exists that effects an alternate pathway. Our kinetic experiments revealed a  $pK_a$  of ~4.9, certainly within error of that seen in the NMR titrations. Curiously, no such  $pK_a$  exists in the analysis of the hydrolysis of cCMP, where the slope is unity (Eftink & Biltonen, 1983).

An 'H-NMR study of RNase A by Rueterjans and coworkers revealed an "abnormally" spread out titration curve between pH 4.5 and 7.0 for His119 in a complex with 3'-dCMP (Haar *et al.*, 1974). They attribute this either to another group titrating in this range, or to "the intrinsic equilibrium constant of the interaction between the histidine-119 and the phosphate group" varying with pH. Knowing of no such group for the first explanation, they chose the latter. It is not clear, however, what is meant by their statement. Perhaps they are suggesting a more complex model involving, for example, a second molecule of inhibitor. A similar anomalous titration was observed for His12 in the complex with 2'-AMP. The fact that in both instances only one histidine is affected suggests that this anomaly is not an artifact resulting from dissociation of the inhibitor. Their results are also consistent with our

observation that the effect of the group with a  $pK_a$  of 5.2 can be felt on either His119 or His12.

In summary, we have found evidence for a second binding site for the inhibitor 3'-UMP, the binding of which affects the binding of the inhibitor at the active site. Further, through global analysis of data for the uncomplexed and complexed proteins, a group with a  $pK_a$  of 5.2 has been observed in the titration of the active site histidines.

		wild-type			D121N			D121A		
residue	[3'-UMP]	pK <sub>a</sub> a	διον	<u> Shigh b</u>	pKa	δίου	<u> </u>	pK <sub>2</sub>	δίων	<u> </u>
His12		5.74±0.03 6.12±0.04 3.99±0.28	8.89±0.03 9.01±0.03	7,67±0.01 8.86	5.84±0.02 6.28±0.06 3.19±1.35	8.96±0.02 8.96±0.11	7.67±0.01 8.86	5.88±0.02 6.32±0.04 2.92±1.87	8.98±0.01 8.97±0.31	7.67±0.01 8.86
His119		5.94±0.02 6.32±0.03	8.82±0.01	7.76±0.01	5.80±0.03 6.24±0.04	8.82±0.01	7.74±0.01	5.80±0.02 6.24±0.03	8.84±0.01	7.74±0.00
His105		6.78±0.01	8.75±0.00	7.70±0.01	6.78±0.01	8.76±0.00	7.70±0.00	6.73±0.01	8.76±0.00	7.71±0.01
His12	1.5 mM	6.33±0.02 6.62±0.07 4.14±0.26	8.75±0.01 8.98±0.02	7.66±0.01 8.86	6.27±0.02 6.49±0.06 4.26±0.15	8.78±0.02 9.05±0.02	7.68±0.05 8.86	6.39±0.01 6.47±0.06 3.78±0.15	8.86±0.01 9.02±0.02	7.68±0.00 8.86
His119	1.5 mM	6.74±0.06 6.86±0.03 4.34±0.59	8.75±0.02 8.91±0.02	7.78±0.01 8.86	6.73±0.06 6.95±0.02 4.63±0.18	8.67±0.02 8.97±0.02	7.74±0.01 8.86	6.37±0.04 6.44±0.03 3.89±0.26	8.73±0.01 9.00±0.02	7.75±0.01 8.86
His105	1.5 mM	6.86±0.01	8.76±0.00	7.70±0.01	6.80±0.01	8.78±0.00	7.71±0.00	6.78±0.01	8.77±0.00	7.70±0.01
His12	11 mM	6.45±0.03 6.35±0.24 3.77±0.36	8.72±0.02 8.99±0.02	7.69±0.01 8.86	6.66±0.01 6.30±0.13 2.63±1.01	8.75±0.04 9.19±1.53	7.71±0.00 8.86	6.80±0.01 6.50±0.05 2.78±0.25	8.78±0.00 9.26±0.15	7.71±0.00 8.86
His119	11 m <b>M</b>	7.95±0.29 7.85±0.07 4.06±0.75	8.69±0.03	7.76±0.03 8.86	7.86±0.15 7.49±0.02 3.88±0.13	8.65±0.01 9.04±0.02	7.74±0.01 8.86	7.18±0.05 6.88±0.02 3.89±0.08	8.64±0.01 9.09±0.01	7.75±0.00 8.86
His105	11 mM	6.86±0.01	8.76±0.00	7.70±0.00	6.87±0.01	8.78±0.00	7.71±0.00	6.79±0.00	8.77±0.00	7.71±0.00
<sup><i>a</i></sup> For His12 and His119, the first $pK_a$ is that observed when the other histidine residue is protonated; the second, when it is										

TABLE 3.1 <sup>1</sup>H-NMR titration of wild-type, D121N, and D121A RNase A

<sup>*a*</sup> For His12 and His119, the first  $pK_a$  is that observed when the other histidine residue is protonated; the second, when it is unprotonated.  $pK_a$ 's listed immediately after these represent perturbations associated with the same. <sup>*b*</sup> The  $\delta_{high}$  values for such perturbations are defined to be 8.86.

Figure 3.1 Chemical shifts of the C<sub>2</sub> protons of His12(●), His119 (O), His105 (■), and His48 (×) of wild-type, D121N, and D121A RNase A (each 3.5 mM) as a function of pH.



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Figure 3.2 Chemical shifts of the C₂ protons of His12(●), His119 (O), His105 (■), and His48 (×) of wild-type, D121N, and D121A RNase A (each 3.5 mM) in the presence of 3'-UMP (1.5 mM) as a function of pH.



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Figure 3.3 Chemical shifts of the C₂ protons of His12 (●), His119 (O), His105 (■), and His48 (×) of wild-type, D121N, and D121A RNase A (each 3.5 mM) in the presence of 3'-UMP (11 mM) as a function of pH.


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Figure 3.4 Schematic presentation of the thermodynamic cube showing the titration of His12, His119, a phosphate of 3'-UMP (in the "flap" of the cube), and a residue we have postulated to be Asp83, in both the free enzyme and the enzyme•inhibitor complex. The nomenclature used is that of equation 1 and the description there of immediately following.



# Chapter 4

## His---Asp Catalytic Dyad of Ribonuclease A: Effect of pH on the Thermal Stability of the Wild-Type, D121N, D121A, and H119A Enzymes

ABSTRACT In native ribonuclease A (RNase A), the sidechains of His119 and Asp121 form a hydrogen bond. The pH-dependencies of the conformational stabilities of wild-type, D121N, D121A, and H119A RNase A were investigated using thermal denaturation over the pH range of 1.2 - 6.0. In all three mutant proteins, the relative loss of stability as a function of pH was the same, while that of wild type was greater by 1.3 kcal/mol. From the pH dependence of  $\Delta G$ , the pKa's of Asp121 in RNase A in the native and the denatured states were calculated to be 2.7 and 3.6, respectively. Further, the conclusion is drawn that His119 is largely, if not solely, responsible for the perturbation of Asp121. The lower stability of D121N RNase A versus wild-type at pH 1.2 of approximately 0.7 kcal/mol is postulated to be due to a bias by His119 towards a conformation in which it can interact with Asp121 versus a position-brought about through rotation about the  $C_{\alpha}$ -C<sub>B</sub> bond-in which it cannot. Of this, 0.2 kcal/mol can be ascribed to the statistical effect of having two sites of similar energy. From the remaining 0.5 kcal/mol,  $K_{eq}$  is calculated to be 2.5. The equivalence in the stability of D121N and D121A RNase A may be due in part to compensating enthalpyentropy changes produced as a result of the loss of a hydrogen bond between D121A and Lys66. The increased stability of H119A RNase A over wild-type at pH 1.2 is partly due to the perturbation of His119 in wild type.

#### INTRODUCTION

The importance of Asp121 to the function of RNase A is suggested by its conservation across the 40 pancreatic ribonucleases of known sequence (Beintema, 1987). Barnard has argued that pancreatic ribonucleases are "essential only in ruminants and certain other herbivores" and thus "can be expected to show wide molecular changes in evolution" (Barnard, 1969). Hence, the conservation of Asp121 is all the more suggestive of its significance. Furthermore, the interaction between Asp121 and His119 defines the motif known as the catalytic dyad—found in lipases and pancreatic ribonucleases—in which a catalytic histidine is hydrogen bonded to an aspartic acid. This motif bears striking similarity to the conserved motif known as the catalytic triad found in serine proteases.

As part of a concerted attack to reveal structure – function relationships in the His-Asp catalytic dyad (see Chapters 2 & 3), the pH-dependence of its conformational stability was determined for the wild-type enzyme and for the D121N, D121A, H119A mutants. The D121N mutant was chosen because it represented a relatively modest change in which many of the hydrogen bonds of Asp121 might be expected to be maintained, including those to one of the two central, catalytic residues—namely His119. The D121A mutant, however, clearly could not maintain any sidechain hydrogen bonds nor interact appreciably with His119. H119A RNase A offered complementary information by eliminating the interaction of the His-Asp interaction without eliminating the sidechain of Asp121.

In this paper, we show that Asp121 is indeed significant to the stability of RNase A. This significance is partially electrostatic, as evidenced by the perturbation of its  $pK_a$  in the native versus denatured protein, and partially due to other factors such as its role in the conformational mobility of His119. We also show that the perturbation of the  $pK_a$  of Asp121 in the wild-type protein is due to its interaction with His119.

#### MATERIALS AND METHODS

#### Materials

Wild-type, D121N, and D121A RNase A were prepared as described in Chapter 2. H119A RNase A was prepared as described (Thompson & Raines, 1994; Thompson, 1995). Buffers used were 30 mM glycine from pH 1.20 to 3.35 and 30 mM acetate from 3.75 to 6.00, both containing 100 mM NaCl. Denaturation curves were determined spectrophotometrically with a protein concentration of approximately 0.7 mg/mL by observing the change in absorbance at 286 nm as the temperature was increased in 1.0 °C increments. The temperature was measured using a thermocouple in the cell block of a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. The cells were sealed with a stopcock so as to inhibit evaporation. The computer was programmed to hold the temperature for 3.0 min. before collecting data to allow for thermal equilibration. At temperatures below approximately 20 °C, it was found necessary to increase progressively this equilibration time.

#### Data Analysis

RNase A unfolding has been shown to approach closely the behavior expected from a twostate system (Tiktopupo & Privalov, 1974; Freire *et al.*, 1990). In a two-state system, the thermodynamic parameters  $\Delta G$ ,  $\Delta H$ ,  $\Delta S$ , and  $\Delta C_p$  of unfolding can be calculated from unfolding curves such as those shown in Figure 4.1 by using the equation:

$$\Delta G = -RT \ln K = -RT \ln \left[ (y_N - y)/(y - y_D) \right] \tag{1}$$

where K is the equilibrium constant, R is the gas constant, T is temperature in Kelvin, y is observed absorbance,  $y_N$  is the absorbance of the native protein and  $y_D$  of the denatured. Generally, both  $y_N$  and  $y_D$  are, to a first approximation, linear functions of temperature.

These functions were obtained by a least-squares analysis of the data in the pre- and posttransitional regions of the denaturation curve. Eq 1 can be solved for y to yield:

$$y = (e^{-\Delta G/RT} y_{\rm D} + y_{\rm N}) / (1 + e^{-\Delta G/RT})$$
(2)

where  $\Delta G(T) = \Delta H_{\rm m} + \Delta C_{\rm p}(T-T_{\rm m}) - T[\Delta S_{\rm m} + \Delta C_{\rm p} \ln (T/T_{\rm m})]$ . The subscript "m" represents the value of the respective thermodynamic constants at the melting point—the point where  $\Delta G = 0$  and K = 1. Thermodynamic parameters were obtained by fitting eq 2 to the denaturation curves using nonlinear least-squares analyses.

It is a thermodynamic necessity that if  $\Delta G$  of denaturation varies as a function of pH, then the pK<sub>z</sub> of a group or groups must be perturbed in the native state versus that of the denatured. The effect these perturbations have on  $\Delta G$  can be calculated using the equation:

$$\Delta G = \Delta G_{[\text{H}_{+}]} = - + RT \sum \ln\{[1 + K_{\text{D},i} / \text{H}^{+}] / [1 + K_{\text{N},i} / \text{H}^{+}]\}$$
(3)

where the summation is over *i* groups with  $K_D$  and  $K_N$  representing dissociation constants in the denatured and native conformations, respectively (Tanford, 1970).

### RESULTS

The variation of  $T_m$  as a function of pH for the four proteins is shown in Figure 4.2. For H119A RNase A, available protein limited us to two pH values: the extremes of pH used in this study of 1.2 and 6.0. Both D121N RNase A and D121A RNase A were seen to have nearly identical  $T_m$ 's throughout the pH range studied, that they are lesser than that of wild type, and that this difference decreases as the pH is lowered. H119A RNase A has a  $T_m$  very near to that of wild-type at pH 6.0 but a significantly higher  $T_m$  at pH 1.2. The drop in  $T_m$ 

for H119A RNase A is similar to the drop observed for D121N and D121A RNase A. The data indicate that titration of all groups is complete by pH 1.6.

Figure 4.3 illustrates the variation of  $\Delta H_m$  versus  $T_m$ . Values of  $\Delta C_p$  were calculated from the slope of a line through these data points. As shown in Table 4.1,  $\Delta C_p$  is similar for all four proteins. The validity of this method in determining  $\Delta C_p$  relies upon the assumption that  $\Delta H_{\text{ionization}}$  is small across the pH range. This assumption has been found to be true, at least at acidic values of pH (Privalov & Khechinashvili, 1974; Becktel & Schellman, 1987).

Attempts to determine  $\Delta C_p$  directly from each denaturation curve were unsuccessful. As shown in Figure 4.4, the error in such determinations was large. Furthermore, at high pH the fits invariably yielded high  $\Delta C_p$  values. Indeed, the plot of  $\Delta C_p$  versus  $T_m$  shown in Figure 4.4 displays a roughly linear relationship. Were this to be the case, Figure 4.3 should show a strong quadratic curvature, which it clearly does not. Attempts to force the high pH curves to the  $\Delta C_p$  values obtained from Figure 4.3 resulted in significant deviation of the data from the fit. As these deviations are most significant just prior to denaturation and as they are in the direction suggestive of "early" denaturation, they may be indicative of some small deviation from the two-state model. That the deviations increase with pH is consistent with the notion of a lower charge on this basic protein promoting aggregation. Alternatively, there may be some curvature in the baseline as has been suggested by others (Tiktopupo & Privalov, 1974; Shortle *et al.*, 1988). Using a baseline with a quadratic term we were indeed able to fit all the data using the  $\Delta C_p$  values in Table 4.1 (data not shown).

With  $\Delta C_p$  in hand,  $\Delta G(T)$  and  $\Delta H(T)$  values can be calculated. A standard temperature of 25 °C was chosen. All thermodynamic parameters referenced to this state are designated as such by the superscript  $^{\emptyset}$ . The results of these calculations for  $\Delta H^{\emptyset}$  are shown in Figure 4.5. The mean values and their standard deviations are found in Table 4.1. The wild-type and D121N enzymes can be seen to be identical within experimental error, as are the D121A and H119A enzymes, while these two sets vary by approximately 7 kcal/mol.

The results of the calculations for  $\Delta G^{\emptyset}$  are shown in Figure 4.6. The curves are qualitatively similar to those found in Figure 4.2 as a result of near identical  $\Delta C_p$  values and relatively small  $\Delta \Delta H^{\emptyset}$  values. The differences seen in D121N and D121A RNase A at high pH are likely within experimental error. It is, however, conceivable that they have a small, long range differential effect on the  $pK_a$  of an ionizable residue. The  $\Delta G^{\emptyset}$  values at pH 1.2 and 6.0 can be found in Table 4.1.

Because of the loss of an imidazolium group in H119A RNase A, this protein would not display a dependence on stability with pH that the other three proteins would if the  $pK_a$  of His119 was perturbed in the native enzyme versus that of the denatured. In order to make comparisons between H119A RNase A and the other three proteins less ambiguous, a correction for the effect that a perturbation of the  $pK_a$  of His119 in the native versus the denatured state would have on the stability of wild-type RNase A was made to H119A RNase A. In other words, the contribution that His119 makes to the pH dependence of the stability of wild-type RNase A was added to that of H119A RNase A so as to produce the  $\Delta G^{\beta}$ 's that would be expected if indeed H119A RNase A did contain an imidazolium group with  $pK_a$ 's identical to that found in wild-type RNase A. This correction was made using Equation 3 and the microscopic  $pK_{a}$ 's determined by NMR titrations of His119 and His12 of wild type (see Chapter 3). Use of the  $pK_a$ 's for His119 and His12 of D121N or D121A RNase A versus those of wild-type resulted in a difference in  $\Delta G^{\emptyset}$  no greater than 0.02 kcal/mol. His12 was included in the calculations as it is known to interact and perturb the  $pK_a$  of His119 (hence the microscopic  $pK_a$ 's). These  $pK_a$ 's are listed in Table 4.1. As a result of using microscopic  $pK_a$  values,  $K_N$  is a function of pH as in the equations:

$$K_{N119}([H^+]) = (K_{119,1} [H^+] + K_{119,2} K_{12,1}) / (K_{12,1} + [H^+])$$
(4)

$$K_{\text{N12}}([\text{H}^+]) = (K_{12,1} [\text{H}^+] + K_{119,2} K_{12,1}) / (K_{119,1} + [\text{H}^+])$$
(5)

The effect of this adjustment to H119A RNase A on  $\Delta G^{\emptyset}$  was to increase the free energy at pH 6.0 by 0.33 kcal/mol and to have no discernible effect at pH 1.2. These values can be found in Table 4.1 in parentheses following the unadjusted values. A similar adjustment was made to wild type to allow direct comparison to H119A RNase A. Here,  $\Delta G^{\emptyset}$  was adjusted so as to eliminate the rise in stability with increasing pH associated with the perturbation of Asp121 in wild type (see the following) followed by eliminating the drop in stability with decreasing pH associated with the perturbation of His119 and His12 in wild type. The attempt here was to adjust wild type so that a comparison between it and H119A RNase A could be made without worrying about differences in charge or perturbed  $pK_4$ 's. The effect of these adjustments was to increase  $\Delta G^{\emptyset}$  at pH 1.2 by 0.38 kcal/mol (largely as the result of the perturbation of Asp121). These values are shown in parentheses in Table 4.1 following the unadjusted results.

The fact that the free energy difference between pH 6.0 and pH 1.2 (the  $\Delta\Delta G^{\emptyset}$ , see Table 4.1) is virtually identically for D121N and D121A RNase A but that for wild-type is larger by approximately 1.3 kcal/mol argues that the difference is due to a perturbation of the pK<sub>a</sub> of Asp121 downwards in the native enzyme from that of the denatured enzyme. To see if we could extract these pK<sub>a</sub> values, Equation 3 was used again. Here, however, we subtracted  $\Delta G^{\emptyset}$  of D121N RNase A from that of wild-type. The  $\Delta G^{\emptyset}$  values so used were calculated using the respective  $\Delta C_p$ 's and  $T_m$ 's with  $\Delta H_m$  coming from regression analysis of  $\Delta H_m$  versus  $T_m$ . The choice between D121N and D121A RNase A was somewhat arbitrary, with the two giving similar results (data not shown). The results of this calculation are shown in Figure 4.7 with the values for the pK<sub>a</sub>'s of Asp121 reported in the legend.

#### DISCUSSION

Approximately two thirds of the loss of stability at pH 6.0 upon substituting Asp121 with either an asparagine or alanine residue can be attributed to the perturbation of Asp121 from a pK<sub>a</sub> of 3.6 in the denatured enzyme to one of 2.7 in the native enzyme. Confirmation of the  $pK_a$  of Asp121 in the native enzyme comes from an NMR study in which the signal of the amide proton of Lys66, which is the most downfield amide in RNase A, is observed to titrate with a  $pK_a$  of approximately 3, which is apparently due to Asp121 (Rico et al., 1989). The remaining 0.7 kcal/mol (seen at pH 1.2) is not so readily explained. A protonated aspartic acid is a close analog to an asparagine. Furthermore, Figure 4.8 shows that only one of the four possible hydrogen bonds is truly inflexible as two of the four hydrogen bonds go to water molecules, and a third goes to a conformationally flexible His119. The inflexible hydrogen bond arises from a hydrogen bond from the backbone of Lys66, with Lys66 donating the hydrogen. For this hydrogen bond to be preserved in D121N RNase A, the oxygen would have to point towards Lys66 and the nitrogen towards the active site, thus preventing any interaction with the protonated His119 and presumably forcing it over into position B. A structural study of a D121N semisynthetic mutant did indeed reveal a shift to position B (deMel et al., 1992). A structural study of D102N trypsin revealed a similar conformational change (Sprang et al., 1987).

The conformational flexibility displayed by His119 has a bias towards position A versus that of B (Table 4.1, pH 1.2). To determine the equilibrium constant for the occupation in positions A and B for His119, allowance must be made for the statistical contribution that two sites make to the stability of the wild-type enzyme. If we assume that His119 only occupies two states (adapted from Cantor & Schimmel, page 854) (Cantor & Schimmel, 1980):

$$1/K_{d,wt} = 1/K_{d,B} + 1/K_{d,A}$$
 (6)

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where  $K_{d,wt}$  represents the equilibrium constant for denaturation of wild-type RNase A,  $K_{d,B}$  represents the equilibrium constant for denaturation of D121N RNase A, and  $K_{d,A}$  represents the equilibrium constant for denaturation that would be observed if His119 only occupied position A. The latter is what we wish to calculate. Using the values for  $\Delta G^{\emptyset}$  at pH 1.2 (Table 4.1),  $K_{d,A} = 0.275$ . From this value, we can calculate  $\Delta G^{\emptyset} = 0.766$  kcal/mol for position A. Comparing this value to  $\Delta G = 0.24$  kcai/rol for D121N RNase A, we observe  $\Delta \Delta G^{\emptyset} = 0.53$  kcal/mol so that  $K_{eq} = [A] / [B] = 2.45$ . This produces a population of 71% in position A to 29% in position B. The difference between the  $\Delta \Delta G^{\emptyset}$  of 0.53 and that calculated by subtracting  $\Delta G^{\emptyset}$  for D121N RNase A from wild-type of 0.73 produces a  $\Delta \Delta \Delta G^{\emptyset}$  cf 0.2 kcal/mol. This value can be ascribed to a statistical effect emanating from the presence of two sites. It is not clear if this preference for site A versus B is due to a favorable hydrogen bond to Asp121 or to lesser conformation strain about the  $C_{\alpha} - C_{\beta}$  bond.

A value of  $K_{eq}$  near unity is consistent with the observation that His119 is often observed in either or both conformations in structural studies and that this equilibrium is readily shifted by inhibitors, solvents, pH, and structural changes (Matthews & Westmorland, 1975; Borkakoti *et al.*, 1982; Rico *et al.*, 1991; deMel *et al.*, 1992; deMel *et al.*, 1994).

If the  $pK_a$  of His119 is higher in position A than in position B as expected from simple electrostatic considerations, then the equilibrium constant between A and B is a function of pH and this equilibrium would shift towards A as the pH is raised to a degree commensurate with the difference in the  $pK_a$ 's. This analysis suggests that the stability the wild-type enzyme gleans from having two states available of similar energy would disappear as the pH was increased and Asp121 was deprotonated. This in turn implies that the  $\Delta G^{\phi}$  observed at high versus low pH is understated by this amount. If the statistical stabilization is subtracted from the  $\Delta G^{\emptyset}$  of wild-type RNase A at low pH, the calculated pK<sub>a</sub> of Asp121 in the native enzyme would be lower by approximately 0.2 pK<sub>a</sub> units to become pK<sub>a</sub> = 2.5.

Quite surprising is the similarity in stability of D121N and D121A RNase A. It is not clear whether or not a water molecule could enter the cavity left by D121A RNase A and thus maintain a hydrogen bond to Lys66. Still, the link that is present in wild type between two somewhat disparate parts of RNase A (with Asp121 in a  $\beta$  sheet linked to Lys66 in a loop) is clearly lost. Rico and coworkers have pointed out the possible significance of this hydrogen bond as being "highly effective in anchoring two loops of the molecule proximal in the space but distant in the sequence" (Rico *et al.*, 1989). The loss of this link may result in a smaller  $\Delta S$  are often accompanied by offsetting changes in  $\Delta H$  (Shortle *et al.*, 1988; Dunitz, 1995). D121A RNase A is observed to have a lower  $\Delta H^{\emptyset}$  by approximately 7 kcal/mol versus that of D121N RNase A, but  $\Delta G^{\emptyset}$  is virtually identical for both. Thus changes in enthalpy are being offset by changes in entropy. Whether the drop in  $\Delta H^{\emptyset}$  observed for D121A RNase A is the result of the loss of a hydrogen bond to Lys66 or whether it is the result of a myriad of smaller enthalpic losses is unclear at this point.

The  $pK_a$  of Asp121 is not perturbed appreciably in the native state from that in the denatured for H119A RNase A. The near identical values of  $\Delta\Delta G^{\emptyset}$  seen for D121N, D121A, and H119A RNase A (adjusted, Table 4.1) is evidence of this. The lack of perturbation of Asp121 in H119A argues that His119 is largely, if not solely, responsible for the perturbation of the  $pK_a$  of Asp121 in wild type of 0.9  $pK_a$  units. This result is somewhat surprising given that the  $pK_a$  of His119 in wild type is greater by only 0.15  $pK_a$  units versus that observed for the D121N and D121A mutants (as determined by NMR titration, Chapter 3).

The increase in stability of H119A RNase A over wild type at pH 1.2 of 0.9 kcal/mol appears to be approximately 50% due to the perturbation of His119 in wild type, as seen

through comparisons of the corrected and uncorrected  $\Delta G^{\phi}$ 's at values of pH of 1.2 and 6.0 for wild-type and for H119A RNase A. The remaining 0.5 kcal/mol is difficult to explain (comparisons between adjusted wild-type and unadjusted H119A RNase A. Table 4.1). The pK, of histidine in the denatured state used in Equation 3 was varied to see how sensitive  $\Delta G^{\emptyset}$  was to this parameter. It was necessary to increase this value to 7.0 from 6.6 to compensate completely for the difference in stability. A value of 7.0 for the  $pK_a$  of histidine in the denatured state seems unrealistically high. So, what could explain the remaining difference? The interaction between protonated Asp121 and protonated His119 suggested by the difference in  $\Delta G^{\emptyset}$ 's for wild-type and D121N RNase A at pH 1.2 (with allowance made for entropic differences generated from differences in the number of available states) would actually argue, if anything, for a decrease in stability. Furthermore, placing a hydrophobic group on the surface of an enzyme would not seem to be a recipe for increased stability as this would not result in reduced solvent accessibility of the hydrophobic group in the native enzyme versus that of the denatured. The latter would increase the stability through the hydrophobic effect. The availability of three staggered conformations of His119 in the denatured state versus two in the native could be the explanation. If the two positions in the native and the three in the denatured all had equal energy, then the denatured state would be favored by 0.24 kcal/mol. Or perhaps the protonated form of His119 is perturbing the  $pK_a$  of yet another group. The perturbation would need to be approximately 0.4  $pK_a$  units or roughly similar to that observed for the His119•His12 interaction. A possible candidate for this is the residue Lys41 which resides in the active site.

The H119A mutant, like the D121A mutant, also displays a reduced  $\Delta H^{\phi}$  versus that of wild-type or D121N RNase A. It also shows compensating enthalpy-entropy changes, with the decrease in  $\Delta H^{\phi}$  being more than offset by a decrease in  $\Delta S^{\phi}$  (which can be viewed as an increase in the entropy of the native enzyme, if one precariously ignores changes in the

denatured state). At this juncture, we lack an hypothesis as to the cause of this enthalpyentropy compensation.

The question of the role of the catalytic dyad, and similarly, the catalytic triad, has been the center of much debate: much of it centered upon its role in catalysis (for a review see (Schowen, 1988). Nevertheless, the possibility that this motif's function may be largely structural should not be ignored. The 2 kcal/mol loss of stability of D121N RNase A versus wild type is a significant percent of the total stability of wild type of 9 kcal/mol. This mutant is particularly illustrative of the stabilization offered through the Asp121...His119 interaction as the D121N mutant would appear to offer little perturbation to the structure versus wild type: especially so in light of the conformational mobility of His119.

In summary, this paper highlights the utility of determining the pH-dependence of protein stability. This determination is particularly important if comparisons are to made between proteins in which an ionizable residue has been replaced with a nonionizable one. The data presented here demonstrate that apparently subtle interactions between residues in a protein can have significant effects on protein stability.

Table 4.1. Thermodynamic Parameters of the Denaturation of Ribonuclease A and Mutants Thereof *					
Protein	$\Delta G^{\phi}_{pH 6.0}^{b}$	$\Delta G^{a}_{pii 1.2}^{b}$	ΔΔG <sup>øc</sup>	$\Delta C_p$	$\Delta H_{ave}^{d}$
WΓ	9.00 (7.86)°	0.97 (1.35) <sup>e</sup>	8.04 (6.51) <sup>e</sup>	1.47	54.8±1.5
D121N	7.06	0.24	6.82	1.53	53.7±1.7
D121A	6.84	0.28	6.56	1.565	46.0±1.7
H119A	8.35 (8.68) <sup>e</sup>	1.88 (1.88)°	6.47 (6.80) <sup>e</sup>	1.40	47.8

<sup>a</sup> Values of  $\Delta G^{\sharp}$  and  $\Delta H^{\sharp}$  are in kcal/mol; values of  $\Delta C_p$  are in kcal/(mol K). <sup>b</sup>  $\Delta G^{\sharp}$  is calculated using the respective  $\Delta C_p$ 's and  $T_m$ 's with  $\Delta H_m$  coming from regression analysis of  $\Delta H_m$  versus  $T_m$ . <sup>c</sup>  $\Delta \Delta G^{\sharp} = \Delta G^{\sharp}_{pH 6.0} - \Delta G^{\sharp}_{pH 1.2}$ . <sup>d</sup>  $\Delta H_{ave}$  represents the mean of all points extrapolated to 25 °C. <sup>e</sup> The values in parentheses were calculated as described in the text using the following pK's for His12 and His119:  $pK_{12,1} = 5.743$ ,  $pK_{12,2} = 6.124$ ,  $pK_{119,1} = 5.936$ ,  $pK_{119,2} = 6.317$ . The value for the pK of histidine in the denatured state was taken as 6.60 (Matthews & Westmorland, 1975).

Figure 4.1 Graph of absorbance versus temperature at pH 2.62. The absorbance levels have been normalized. The lines represent the nonlinear least-squares fits of the data as described in the text.



Figure 4.2 Graph of  $T_m$  versus pH for wild-type (O), D121N ( $\Box$ ), D121A ( $\times$ ), and H119A RNase A (+). The lines simply connect the points.



Figure 4.3 Graph of  $\Delta H_m$  versus  $T_m$  for wild-type (O), D121N ( $\Box$ ), D121A ( $\times$ ), and H119A RNase A (+). The lines represent linear least-squares fits of the data, except of H119A wherein two point define a line. The values of the slope of the lines (the  $\Delta C_p$ 's) are given in Table 4.1.



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Figure 4.4 Graph of  $\Delta C_p$  versus  $T_m$  for wild-type (O), D121N ( $\Box$ ), D121A ( $\times$ ), and H119A RNase A (+). The line represents a linear least-squares fit of all the data.



Figure 4.5 Graph of ΔH<sup>Ø</sup> versus T<sub>m</sub> for wild-type (O), D121N (□), D121A RNase A
(×). The lines represent the mean average of the data. The values of the average as well as the standard deviations are given in Table 4.1.

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Figure 4.6 Graph of  $\Delta G^{\emptyset}$  versus pH for wild-type (O), D121N ( $\Box$ ), D121A ( $\times$ ), H119A RNase A (+). The points were calculated as described in the text using the observed  $\Delta H_{\rm m}$  values. The lines are connected points which were calculated using  $\Delta H_{\rm m}$  values from regression analysis of  $\Delta H_{\rm m}$  versus  $T_{\rm m}$ .



Figure 4.7 Graph of  $\Delta\Delta G^{\emptyset}$  ( $\Delta G^{\emptyset}$  wild-type -  $\Delta G^{\emptyset}$ D121N) versus pH ( $\bullet$ ) where  $\Delta G^{\emptyset}$  was calculated using  $\Delta H_{\rm m}$  values from regression analysis of  $\Delta H_{\rm m}$  versus  $T_{\rm m}$ . The line represents the nonlinear least-squares fit of the data as described in the text. The parameters for the fit are as follows:  $pK_{a,{\rm D}} = 3.55$ ,  $pK_{a,{\rm N}} = 2.68$ ,  $\Delta G^{\emptyset}_{\rm [H+]==} = 0.78$  kcal/mol.



Figure 4.8 Three-dimensional structure of RNase A depicting the geometry of Asp121 and surrounding residues (Borkakoti *et al.*, 1982). The dashes represent putative hydrogen bonds.



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