

# His $\cdots$ Asp Catalytic Dyad of Ribonuclease A: Conformational Stability of the Wild-Type, D121N, D121A, and H119A Enzymes<sup>†</sup>

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**ABSTRACT:** Residue His119 acts as an acid/base during the cleavage/hydrolysis reactions catalyzed by bovine pancreatic ribonuclease A (RNase A). In the native enzyme, His119 forms a hydrogen bond with Asp121. This His $\cdots$ Asp dyad is conserved in all homologous pancreatic ribonucleases of known sequence. Yet, replacing Asp121 with an asparagine or alanine residue does not have a substantial effect on either structure or function [Schultz, L. W., Quirk, D. J., and Raines, R. T. (1998) *Biochemistry* 37, 8886–8898]. Here, the pH dependencies of the conformational stabilities of wild-type RNase A and the D121N, D121A, and H119A variants were determined by monitoring thermal stability over the pH range 1.2–6.0. Replacing Asp121 with an asparagine or alanine residue results in a loss of conformational stability at pH 6.0 of  $\Delta\Delta G^\circ = 2.0$  kcal/mol, from a total of 9.0 kcal/mol. The magnitude of this loss is similar to that to transition-state binding during catalysis. As the pH decreases, the aspartate residue becomes protonated and  $\Delta\Delta G^\circ$  decreases. D121N RNase A and D121A RNase A are approximately equivalent in conformational stability. This equivalence arises from compensating changes to enthalpy and entropy. A general analytical method was developed to determine the value of the  $pK_a$  of a residue in the native and denatured states of a protein by comparing the pH–stability profile of the wild-type protein with that of a variant in which the ionizable residue is replaced with a nonionizable one. Accordingly, Asp121 was found to have  $pK_a$  values of approximately 2.4 and 3.4 in the native and denatured states, respectively, of wild-type RNase A. This change in  $pK_a$  can account fully for the differential effects of pH on the conformational stabilities of the wild-type and variant proteins. We conclude that the His $\cdots$ Asp catalytic dyad in pancreatic ribonucleases has two significant roles: (1) to position the proper tautomer of His119 for catalysis and (2) to enhance the conformational stability of the native enzyme. Most enzymic residues contribute to catalysis or stability (or neither). Asp121 of RNase A is a rare example of a residue that contributes equally to both.

The ability of a protein to perform a biological function relies on the stability of its native three-dimensional structure. The forces that maintain this three-dimensional structure are the same as those that enable proteins to bind ligands or catalyze reactions (1). Accordingly, amino acid residues that contribute to protein function could also contribute to protein stability. Yet, few residues have been recognized as playing these dual roles.

Hydrogen-bonded histidine and aspartate residues are found in the active sites of many enzymes. For example, the His $\cdots$ Asp motif is found in the active sites of serine carboxypeptidase (2), acetylcholinesterase (3), phospholipase A<sub>2</sub> (4), haloalkane dehalogenase (5), diene lactone hydrolase (6), and a variety of zinc-dependent enzymes (7). This motif is also a component of the renowned catalytic triad found in the active sites of serine proteases (8–10).

In bovine pancreatic ribonuclease A [RNase A<sup>1</sup> (11); EC 3.1.27.5], the active-site histidine and aspartate residues are

His119 and Asp121 (Figure 1). The results of site-directed mutagenesis experiments have shown that His119 has a fundamental role: to act as an acid during catalysis of RNA cleavage (15, 16). The importance of Asp121 can be inferred from its conservation in all of the over forty homologous pancreatic ribonucleases of known sequence (17, 18).

We are interested in structure–function relationships in the His $\cdots$ Asp catalytic dyad of RNase A. Previously, we reported that replacing Asp121 with an asparagine or alanine residue has no effect on the overall three-dimensional structure of the enzyme, and a significant but not substantial effect on catalysis (14). These results belie the conservation of the His $\cdots$ Asp dyad in pancreatic ribonucleases.

What then is the role of the His $\cdots$ Asp catalytic dyad of RNase A? The proton is the least sterically demanding perturbant of protein structure. pH–rate profiles have been used often and with great success to reveal the role of ionizable residues in enzymatic catalysis (19, 20). Similarly, pH–stability profiles can in theory be used to reveal the contribution of such residues to conformational stability. Here, we have analyzed in detail the pH dependency of the conformational stabilities of wild-type RNase A and the

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<sup>1</sup> Abbreviations: A, absorbance; ppm, parts per million; RNase A, bovine pancreatic ribonuclease A; UV, ultraviolet.

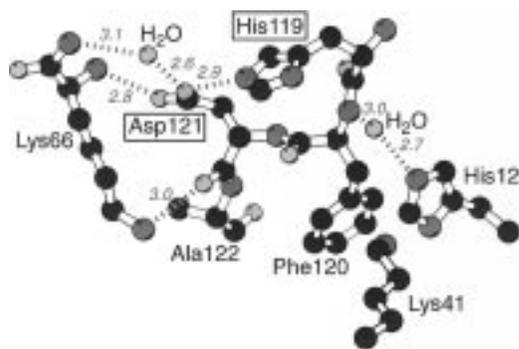


FIGURE 1: Crystalline structure of the active site of ribonuclease A. This figure was created with the program MOLSCRIPT v2.2 (13) using coordinates determined at 1.26 Å by X-ray diffraction analysis [PDB entry 7rsa (12)]. Hydrogen bonds of  $\leq 3.2$  Å are labeled. The crystalline structures of D121N RNase A [3rsd (14)] and D121A RNase A [4rsd (14)] are similar, aside from residue 121.

D121N, D121A, and H119A variants. We find that Asp121 makes a significant contribution to the conformational stability of native RNase A. This finding reveals a previously unappreciated role for the prevalent His $\cdots$ Asp dyad.

## EXPERIMENTAL PROCEDURES

**Materials.** The construction of a bovine pancreatic cDNA library, the cloning of the cDNA that codes for RNase A, the efficient expression of this cDNA in *Escherichia coli* (21), and the preparation and purification of wild-type RNase A and the D121N and D121A variants (14) were described previously. The preparation and purification of H119A RNase A was also described previously (16, 22).

**Thermal Denaturation.** Protein denaturation was assessed by ultraviolet (UV) spectroscopy. The change in absorbance at 286 nm ( $A_{286}$ ) was monitored 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. To allow for thermal equilibration, we programmed the spectrophotometer to maintain temperatures for 3.0 min before collecting data. At temperatures below 20 °C, it was necessary to increase progressively this equilibration time. Samples (1.8 mL) contained protein (0.7 mg/mL; 0.05 mM), NaCl (0.10 M), and either 30 mM glycine-HCl buffer (pH 1.20–3.35) or 30 mM sodium acetate buffer (pH 3.75–6.00). The pH range 1.20–6.00 was chosen to encompass the titration of an aspartic acid residue. Cuvettes containing samples were sealed with a stopcock to prevent evaporation during thermal denaturation experiments.

**Data Analysis.** The unfolding of RNase A has been shown to approach closely the behavior expected from a two-state system (23, 24). In a two-state system, the value of  $\Delta G$  for the unfolding transition can be calculated from denaturation curves by using the following equation (25):

$$\Delta G = -RT \ln K = -RT \ln \frac{y_N - y}{y - y_U} \quad (1)$$

where  $K$  is the equilibrium constant,  $R$  is the gas constant,  $T$  is the temperature in K,  $y$  is observed absorbance,  $y_N$  is the

absorbance of the native protein, and  $y_U$  is the absorbance of the unfolded protein. In general, both  $y_N$  and  $y_U$  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 post-transitional regions of the denaturation curve. Equation 1 can be solved for  $y$  to yield the following:

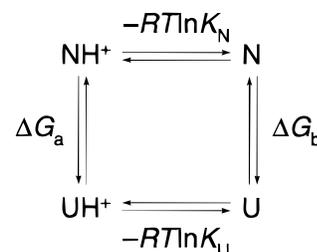
$$y = \frac{e^{-\Delta G/RT} y_U + y_N}{1 + e^{-\Delta G/RT}} \quad (2)$$

with (25, 26)

$$\Delta G(T) = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left(T_m - T + T \ln \frac{T}{T_m}\right) \quad (3)$$

In eq 3, the subscript “m” refers to the value of the thermodynamic constant at the melting temperature ( $T_m$ ), which is the temperature at the midpoint of the thermal denaturation curve where  $K = 1$  and  $\Delta G = 0$ . Thermodynamic parameters were obtained by fitting eqs 2 and 3 to the denaturation curves using nonlinear least-squares analysis.

If  $\Delta G$  of denaturation varies as a function of pH, then it is a thermodynamic necessity that the  $pK_a$  of one or more functional groups differ in the native and unfolded proteins (27). This requirement is apparent in a scheme drawn for a protein with only one ionizable group:



where “N” and “U” refer to the native and unfolded states of the protein, respectively, and  $\Delta G_a$  and  $\Delta G_b$  refer to the denaturation of the acidic and basic forms of the protein, respectively. If  $\Delta G_a \neq \Delta G_b$ , then  $pK_N \neq pK_U$ . The effect of pH on conformational stability is the following:

$$\Delta G([\text{H}^+]) = -RT \ln \frac{[\text{UH}^+] + [\text{U}]}{[\text{NH}^+] + [\text{N}]} \quad (4)$$

with the effect of  $pK_N$  and  $pK_U$  on this pH dependence being (28)

$$\Delta G([\text{H}^+]) = \Delta G([\text{H}^+] = \infty) + RT \ln \left( \frac{1 + \frac{K_N}{[\text{H}^+]}}{1 + \frac{K_U}{[\text{H}^+]}} \right) \quad (5)$$

Of course, most proteins have many ionizable groups, and the more general form of eq 5 is the following (28):

$$\Delta G([\text{H}^+]) = \Delta G([\text{H}^+] = \infty) + RT \sum_{i=1}^n \ln \left( \frac{1 + \frac{K_{N,i}}{[\text{H}^+]}}{1 + \frac{K_{U,i}}{[\text{H}^+]}} \right) \quad (6)$$

where the summation is over  $n$  groups with  $K_{N,i}$  and  $K_{U,i}$  representing the acid dissociation constants of each group in the native and unfolded conformations, respectively.

The values of  $K_N$  and  $K_U$  for a particular ionizable group can in theory be determined by comparing the pH dependency of the conformational stability of the wild-type protein with that of a variant in which the ionizable group is replaced with a nonionizable group. In the variant protein, eq 6 becomes the following:

$$\Delta G([\text{H}^+]) = \Delta G([\text{H}^+] = \infty) + RT \sum_{i=1}^{n-1} \ln \left( \frac{1 + \frac{K_{N,i}}{[\text{H}^+]}}{1 + \frac{K_{U,i}}{[\text{H}^+]}} \right) \quad (7)$$

where the summation is now over  $n - 1$ , rather than  $n$ , groups. Subtracting eq 7 from eq 6 yields

$$\Delta \Delta G([\text{H}^+]) = \Delta \Delta G([\text{H}^+] = \infty) + RT \ln \left( \frac{1 + \frac{K_N}{[\text{H}^+]}}{1 + \frac{K_U}{[\text{H}^+]}} \right) \quad (8)$$

where  $\Delta \Delta G([\text{H}^+])$  is the difference in the pH dependencies of the wild-type and variant proteins. We have made an assumption in deriving eq 8: the effect of replacing an ionizable group on the pH dependence of conformational stability is due to the ionizable group of interest, and not to  $pK_a$  perturbations of other ionizable groups. Equation 8 will be reliable (1) if any such perturbations are small, or (2) if any such perturbations are to groups with  $pK_a$  values beyond the range of the pH titration. Then, replacing an ionizable residue in a wild-type protein (e.g., Asp121 in RNase A) with a nonionizable residue (e.g., asparagine or alanine) enables the values of  $K_N$  and  $K_U$  for the residue to be determined by fitting the data in a plot of  $\Delta \Delta G$  versus pH to eq 8.

## RESULTS

Typical denaturation curves for wild-type RNase A and the D121N, D121A, and H119A variants are shown in Figure 2. Values of  $T_m$  were calculated from thirteen such curves for each of the four proteins, and the variations of  $T_m$  as a function of pH are shown in Figure 3. Values of  $T_m$  from replicate experiments differed by less than 0.1 °C. D121N RNase A and D121A RNase A were seen to have nearly identical  $T_m$  values throughout the pH range studied. These  $T_m$  values are less than those of the wild-type protein, but the deviation decreases as the pH is lowered. The  $T_m$  of the H119A variant is close to that of wild-type RNase A at pH 6.0 but is significantly greater at pH 1.2.

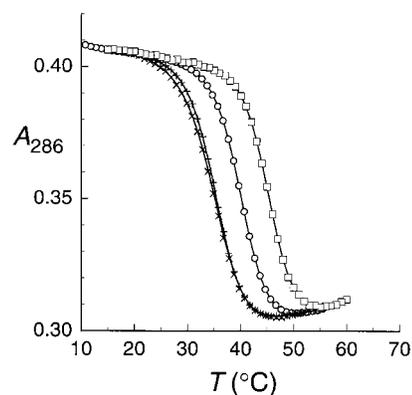


FIGURE 2: Plot of absorbance at 286 nm versus temperature at pH 2.62 for wild-type ribonuclease A (O) and the D121N (x), D121A (+), and H119A (□) variants. Solutions consisted of 30 mM glycine-HCl buffer (pH 2.62) containing protein (0.7 mg/mL; 0.05 mM) and NaCl (0.10 M). For easier comparison, the absorbance levels of the variants are normalized to that of wild-type RNase A. The lines are nonlinear least-squares fits of the data to eq 1.

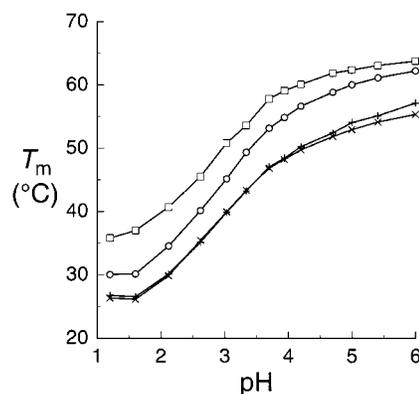


FIGURE 3: Plot of  $T_m$  versus pH for wild-type ribonuclease A (O) and the D121N (x), D121A (+), and H119A (□) variants. Values of  $T_m$  from replicate determinations varied by less than 0.1 °C. The lines merely connect the points.

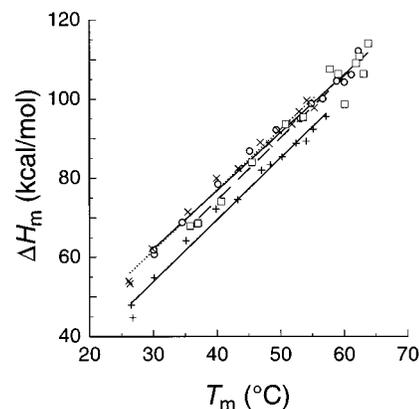


FIGURE 4: Plot of  $\Delta H_m$  versus  $T_m$  for wild-type ribonuclease A (O) and the D121N (x), D121A (+), and H119A (□) variants. The lines are linear least-squares fits of the data. Values  $\pm$  SE of the slopes (that is,  $\Delta C_p$ ) are listed in Table 1.

The variation of  $\Delta H_m$  with  $T_m$  for the four proteins is shown in Figure 4. Values of  $\Delta C_p$  were calculated from the slope of the line through each set of data points. As listed in Table 1, the  $\Delta C_p$  values are 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 valid, at least at low values

Table 1: Thermodynamic Parameters for the Conformational Stabilities of Wild-Type RNase A, D121N RNase A, D121A RNase A, and H119A RNase A at 25 °C

RNase A	$\Delta G^\circ_{\text{pH}6.0^a}$ (kcal/mol)	$\Delta G^\circ_{\text{pH}1.2^a}$ (kcal/mol)	$\Delta\Delta G^\circ^b$ (kcal/mol)	$\Delta C_p^c$ [kcal/(mol·K)]	$\Delta H^\circ^c$ (kcal/mol)
wild-type	9.0 ± 0.1	1.0 ± 0.1	8.0 ± 0.2	1.47 ± 0.04	54.8 ± 1.0
D121N	7.1 ± 0.1	0.24 ± 0.01	6.9 ± 0.1	1.54 ± 0.05	53.7 ± 0.8
D121A	6.8 ± 0.1	0.28 ± 0.01	6.5 ± 0.1	1.57 ± 0.05	46.0 ± 0.9
H119A	9.2 ± 0.2	2.1 ± 0.1	7.1 ± 0.3	1.57 ± 0.09	51 ± 3

<sup>a</sup> Values ± SE of  $\Delta G^\circ$  were calculated at 25 °C from values of  $\Delta C_p$ ,  $T_m$ , and  $\Delta H_m$  using eq 3. <sup>b</sup>  $\Delta\Delta G^\circ = \Delta G^\circ_{\text{pH}6.0} - \Delta G^\circ_{\text{pH}1.2}$ . <sup>c</sup> Values ± SE of  $\Delta C_p$  and  $\Delta H^\circ$  were calculated at 25 °C by linear least-squares fit of the data in Figure 4.

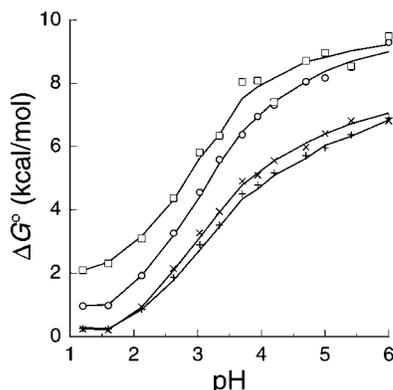


FIGURE 5: Plot of  $\Delta G^\circ$  versus pH for wild-type ribonuclease A (○) and the D121N (×), D121A (+), and H119A (□) variants. The points were calculated from the observed  $\Delta H_m$  values using eq 3. The lines were calculated by using  $\Delta H_m$  values from regression analysis of  $\Delta H_m$  versus  $T_m$ .

of pH (26, 29). Moreover, the value of  $\Delta C_p$  for the denaturation of RNase A has been found to be independent of pH (29–31).

With the value of  $\Delta C_p$  in hand, the value of  $\Delta G(T)$  can be calculated with eq 3. A standard temperature of 25 °C was chosen, and all thermodynamic parameters at this temperature are designated by a “°”. The values of  $\Delta G^\circ$  thus calculated are shown in Figure 5. The curves in Figure 5 are similar to those in Figure 3 as a result of nearly identical  $\Delta C_p$  values and relatively small  $\Delta\Delta H^\circ$  values. The values of  $\Delta G^\circ$  at pH 1.2 and 6.0 are listed in Table 1.

Decreasing the pH from 6.0 to 1.2 decreases the conformational stability of wild-type RNase A and all three variants (Figure 5). The effect of pH is largest for the wild-type protein, and decreases in the following order (Table 1): wild-type RNase A ( $\Delta\Delta G^\circ = 8.0$  kcal/mol) > H119A RNase A (7.1) > D121N RNase A (6.9) > D121A RNase A (6.5). Hence, the changes to the His•••Asp dyad have effects on the pH-dependent conformational stability that range from 0.9 to 1.5 kcal/mol.

The pH–stability data can be used to derive the  $pK_a$  values of Asp121 in the native state and denatured state of wild-type RNase A. To extract these  $pK_a$  values, we used eq 8. The use of eq 8 is justified because no side chains proximal to Asp121 titrate between pH 1.2 and 6.0, except for those of His12 and His119 (Figure 1), and titrations monitored by <sup>1</sup>H NMR spectroscopy reveal that replacing Asp121 with an asparagine or alanine residue changes the microscopic  $pK_a$  values of His12 and His119 by less than 0.2 units (32). Values of  $\Delta\Delta G^\circ$  were obtained by subtracting the values of  $\Delta G^\circ$  of the D121N or D121A variant from that of the wild-type protein. The results of this calculation are shown in Figure 6. Data on the conformational stability of the D121N

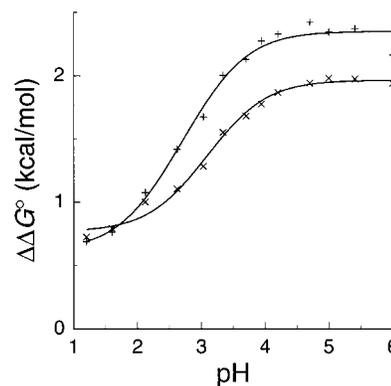


FIGURE 6: Plot of  $\Delta\Delta G^\circ (= \Delta G^\circ_{\text{wild-type}} - \Delta G^\circ_{\text{D121N/A}})$  versus pH for D121N ribonuclease A (×) and D121A ribonuclease A (+). Values of  $\Delta G^\circ$  were calculated from values of  $\Delta H_m$  using regression analysis of  $\Delta H_m$  versus  $T_m$ . The lines are nonlinear least-squares fits of the data to eq 8, using the parameters  $pK_U = 3.5$ ,  $pK_N = 2.6$ , and  $\Delta\Delta G^\circ([H^+] = \infty) = 0.78$  kcal/mol for D121N RNase A; and  $pK_U = 3.3$ ,  $pK_N = 2.1$ , and  $\Delta\Delta G^\circ([H^+] = \infty) = 0.62$  kcal/mol for D121A RNase A.

variant indicates that the  $pK_a$  values of Asp121 are 2.6 in native RNase A and 3.5 in the denatured protein. Data on the D121A variant indicates that the  $pK_a$  values of Asp121 are 2.1 in native RNase A and 3.3 in the denatured protein. Thus, the pH–stability profiles revealed that the  $pK_a$  of Asp121 decreases by approximately one unit upon the unfolding of wild-type RNase A.

## DISCUSSION

**Conformational Stability.** Hydrogen bonds between proximal side chains can stabilize  $\alpha$ -helices (33–35) and  $\beta$ -sheets (36). In native RNase A, His119 and Asp121 are on the  $\beta_4$  strand of a twisted, four-stranded, antiparallel  $\beta$ -sheet. Both of these residues have ionizable side chains. Previously, we reported on the pH–rate profile of catalysis by wild-type RNase A and variants at Asp121 (14). We found that the changes had no effect on the pH dependence of catalysis. Here, we have determined the pH–stability profiles of wild-type RNase A and variants at His119 and Asp121. Our results indicate that the interaction between these two residues enhances significantly the conformational stability of RNase A.

The  $pK_a$  of the side-chain carboxyl group of Asp121 is perturbed from approximately 3.4 (which is the average of the values calculated from the data in Figure 6) in the denatured protein to approximately 2.4 in the native protein. The  $pK_a$  of 3.4 in denatured RNase A is close to the  $pK_a$  of 3.9 observed for the aspartic acid side chain in Gly<sub>2</sub>AspGly<sub>2</sub> (37). The lower  $pK_a$  of 2.4 in native RNase A is consistent with expectations from the three-dimensional structure of the

protein. In crystalline RNase A, the side-chain  $O_{\delta 2}$  of Asp121 forms a hydrogen bond of 2.8 Å with the main-chain nitrogen of Lys66 (12). In  $^1\text{H}$  NMR experiments performed under solution conditions different than those used herein, the signal of the main-chain NH of Lys66 has been observed to titrate with a  $pK_a$  of 2.94 (38) or 3.1 (39). This titration could report on the deprotonation of the carboxyl group of Asp121.

The perturbation to the  $pK_a$  of Asp121 could be a result of the conformational stability conferred by Asp121. The values of  $\Delta\Delta G^\circ$  listed in Table 1 report on the pH-induced loss of conformational stability. These values for the D121N and D121A variants are lower by 1.1 and 1.5 kcal/mol, respectively, than that for wild-type RNase A. From eq 1, the change in free energy that corresponds to a change in  $pK_a$  at 25 °C is  $\Delta\Delta G^\circ = (1.4 \text{ kcal/mol})\Delta pK_a$ . Thus, the observed loss of conformational stability of 1.1–1.5 kcal/mol corresponds to a change of approximately one  $pK_a$  unit, and one unit is the change observed in the  $pK_a$  of Asp121 upon folding of the wild-type protein. In other words, depressing the  $pK_a$  of Asp121 can account fully for the differential effects of pH on the conformational stabilities of the wild-type and variant proteins.

The side chain of Asp121 can simultaneously form two hydrogen bonds: one with the side-chain  $N^{\epsilon 1}$  of His119 and another with the main-chain nitrogen of Lys66. The hydrogen bond between the side chain of Asp121 and the main chain of Lys66 restricts conformational entropy by linking two parts of the native protein that are distal in amino acid sequence. However, because the side chain of Asp121 is anionic and the side chain of His119 is cationic near neutral pH, the hydrogen bond with His119 is likely to be stronger than that with the uncharged main chain of Lys66 (40). Still, the  $^1\text{H}$  chemical shift of the main-chain NH of Lys66 does decrease by >2 ppm as Asp121 is protonated (38). This large change in chemical shift suggests that the interaction between Asp121 and Lys66 is considerable.

In D121N RNase A, the interaction between Asn121 and Lys66 is weak. A hydrogen bond between the side-chain oxygen of Asn121 and the main-chain nitrogen of Lys66 is evident in the structure of crystalline D121N RNase A (14). The side chain of Ala121 in D121A RNase A cannot form such a hydrogen bond. Yet, the conformational stabilities of the D121N and D121A variants are similar at all values of pH (Figure 5). This finding suggests that the hydrogen bond between the side chain of Asn121 and the main chain of Lys66 makes only a small contribution to conformational stability.

Replacing Asp121 with an asparagine or alanine residue results in changes in conformational enthalpy that are offset by changes in conformational entropy. The value of  $\Delta H^\circ$  for D121A RNase A is lower by approximately 8 kcal/mol than that for D121N RNase A. Yet,  $\Delta G^\circ (= \Delta H^\circ - T\Delta S^\circ)$  is virtually identical for the two variants. Such enthalpy–entropy compensation is a common feature of biopolymer structure–function relationships (41, 42). Differential solvation and the flexibility of the side chain of His119 (vide infra) are likely to contribute to the enthalpy–entropy compensation observed herein.

The values of  $\Delta G^\circ$  for the wild-type and variant enzymes are consistent with expectations based on Coulombic interactions within their active sites. The protonation states at pH 1.2 and 6.0 of the His•••Asp dyad are shown in Figure 7.

RNase A	pH 1.2 $\Delta G^\circ$			pH 6.0 $\Delta G^\circ$		
	12	119	121	12	119	121
Wild-Type	+	+	○	+	+	−
D121(N/A)	+	+	−0.2	+	+	−7.0
H119A	+	○	2.1	+	−	9.3

FIGURE 7: Notional depiction of Coulombic interactions in the active sites of wild-type RNase A and the D121N, D121A, and H119A variants at pH 1.2 and 6.0. The residues depicted are those with side chains that titrate significantly between pH 1.2 and 6.0. His12 and His119 are more cationic (larger “+” sign) at pH 1.2 than at pH 6.0. Values of  $\Delta G^\circ$  are from Table 1.

We have also included His12 in this analysis because its side chain is proximal to the His•••Asp dyad in native RNase A (Figure 1).<sup>2</sup> Only H119A RNase A does not experience unfavorable Coulombic interactions within its active site, and that enzyme has the most conformational stability of the four at all values of pH between 1.2 and 6.0. At pH 6.0, only the D121N and D121A enzymes have positive charges that are not compensated by a negative charge, and those enzymes have the least conformational stability of the four. Coulombic interactions in the active site of wild-type RNase A are similar to those of the D121N and D121A enzymes at pH 1.2 and to those of the H119A enzyme at pH 6.0 (Figure 7), and its value of  $\Delta G^\circ$  varies accordingly (Figure 5).

*Conformational Equilibria.* A dynamic equilibrium exists between two rotamers of the side chain of His119 (43–45).<sup>3</sup> In position A,  $N_{\epsilon 2}$  of His119 forms a hydrogen bond with  $O_{\delta 1}$  of Asp121 (Figure 1), with the side chain of His119 having torsion angles of  $\chi_1 = 149^\circ$  and  $\chi_2 = -101^\circ$ . In position B, the imidazolyl group is removed from Asp121 by 7 Å, with  $\chi_1 = -69^\circ$  and  $\chi_2 = -63^\circ$ . In analyses of the crystalline and solution structures of RNase A, His119 has been found in position A, position B, or both (for reviews, see refs 46, 47). These structural studies indicate that the value of the equilibrium constant for the  $B \rightleftharpoons A$  interconversion is approximately  $K = [A]/[B] = 4$  near neutral pH.

Our pH–stability profiles enable us to estimate the value of the  $B \rightleftharpoons A$  equilibrium constant at low pH. A protonated aspartate residue is both an isoelectronic and an isoprotonic analogue of an asparagine. However, these two residues differ in that both side-chain oxygens of an aspartate residue (at low or high pH) can accept a hydrogen bond, but only the side-chain oxygen (and not the side-chain nitrogen) of an asparagine residue can accept a hydrogen bond. Replacing Asp121 with an asparagine residue decreases by 1.9 kcal/mol the conformational stability of RNase A at pH 6.0, and 0.8 kcal/mol of this decrease remains at pH 1.2 (Table 1). We demonstrated previously that the main-chain nitrogen of Lys66 donates a hydrogen bond to a side-chain oxygen of both Asp121 in wild-type RNase A and Asn121 in D121N RNase A (14). As a result, the side-chain nitrogen of Asn121 faces the active site. This orientation prevents the formation of a hydrogen bond with the protonated (that is, imidazolium)

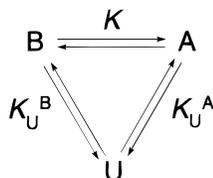
<sup>2</sup> We have excluded Lys41 and Lys66 from this analysis because all existing data indicate that their side-chain amino groups have  $pK_a \gg 6$ . Accordingly, the presence of Lys41 and Lys66 cannot explain changes in conformational stability between different proteins at pH 1.2–6.0.

<sup>3</sup> The active-site histidine residue in carbonic anhydrase II undergoes a similar rotation (42).

form of His119, presumably forcing the side chain of protonated His119 to reside exclusively in position B. In contrast, the protonated form of His119 in wild-type RNase A could be in either position A or position B and still form a hydrogen bond with the side chain of Asp121. We can therefore dissect the  $B \rightleftharpoons A$  equilibrium as follows. If the side chain of His119 exists in only two states, then the following is true (48):

$$\frac{1}{K_U} = \frac{1}{K_U^A} + \frac{1}{K_U^B} \quad (9)$$

where  $K_U$  is the equilibrium constant for the denaturation of wild-type RNase A,  $K_U^A$  is the equilibrium constant for the denaturation of wild-type RNase A if His119 occupied only position A, and  $K_U^B$  is the equilibrium constant for the denaturation of wild-type RNase A if His119 occupied only position B (as in D121N RNase A at pH 1.2). The values of  $\Delta G^\circ_{\text{pH}1.2}$  for wild-type and D121N RNase A (Table 1) can be used to calculate that  $K_U = 0.19$  and  $K_U^B = 0.67$  at pH 1.2. Then by eq 9,  $K_U^A = 0.27$ . If we assume that wild-type RNase A can denature with His119 in either position A or position B, then  $K_U^A$  and  $K_U^B$  are related to  $K = [A]/[B]$  as in the following scheme:



where “A” and “B” refer to the native state of wild-type RNase A with His119 in position A and position B, respectively, and “U” refers to the unfolded state of wild-type RNase A. From the values of  $K_U^A = 0.27$  and  $K_U^B = 0.67$ , we calculate that  $K_{AB} = K_U^B/K_U^A = 2.5$  at pH 1.2.

Taken together, these results paint the following picture. At pH 1.2, His119 occupies position A 2.5 times more often than it does position B. As the pH is increased, Asp121 is deprotonated and the  $B \rightleftharpoons A$  equilibrium shifts even further toward position A. Additional increases in pH bring about the deprotonation of His119. The concomitant loss of favorable Coulombic interactions likely shifts the  $B \rightleftharpoons A$  equilibrium away from position A, with  $K = [A]/[B] = 4$  near neutral pH (46, 47).

**Conclusions.** The interaction between the histidine and aspartate residues of the Ser $\cdots$ His $\cdots$ Asp catalytic triad has been at the center of much debate among biochemists (8–10). This debate has focused on the role of the His $\cdots$ Asp interaction in catalysis. Yet, when Asp121 of the His $\cdots$ Asp catalytic dyad of RNase A is replaced with an asparagine or alanine residue, the enzyme loses 2.0 kcal/mol of its 9.0 kcal/mol of conformational stability. This decrease in  $\Delta G^\circ$  is similar to the loss to transition-state binding during catalysis of RNA cleavage (14) as well as to the loss to stability from analogous replacements in other proteins (4, 49–52). We therefore conclude that a major role of the His $\cdots$ Asp catalytic dyad of RNase A, and perhaps that of other enzymes, is to enhance the conformational stability of the enzyme.

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