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His · · · Asp Catalytic Dyad of Ribonuclease A: Histidine pK_a Values in the Wild-Type, D121N, and D121A Enzymes

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ABSTRACT Bovine pancreatic ribonuclease A (RNase A) has a conserved His · · · Asp catalytic dyad in its active site. Structural analyses had indicated that Asp^{121} forms a hydrogen bond with His^{119}, which serves as an acid during catalysis of RNA cleavage. The enzyme contains three other histidine residues including His^{12}, which is also in the active site. Here, ^{1}H-NMR spectra of wild-type RNase A and the D121N and D121A variants were analyzed thoroughly as a function of pH. The effect of replacing Asp^{121} on the microscopic pK_a values of the histidine residues is modest: none change by more than 0.2 units. There is no evidence for the formation of a low-barrier hydrogen bond between His^{119} and either an aspartate or an asparagine residue at position 121. In the presence of the reaction product, uridine 3′-phosphate (3′-UMP), protonation of one active-site histidine residue *favors* protonation of the other. This finding is consistent with the phosphoryl group of 3′-UMP interacting more strongly with the two active-site histidine residues when both are protonated. Comparison of the titration curves of the unliganded enzyme with that obtained in the presence of different concentrations of 3′-UMP shows that a second molecule of 3′-UMP can bind to the enzyme. Together, the data indicate that the aspartate residue in the His · · · Asp catalytic dyad of RNase A has a measurable but modest effect on the ionization of the adjacent histidine residue.

INTRODUCTION

RNase A (EC 3.1.27.5) has been the object of much landmark work in biochemistry and biophysics (Raines, 1998). For example, RNase A was the first enzyme and second protein (after insulin) for which a complete amino acid sequence was determined (Smythe et al., 1963), and the third enzyme and fourth protein (after myoglobin, lysozyme, and carboxypeptidase) whose structure was solved by x-ray diffraction analysis (Kartha et al., 1967). The use of NMR spectroscopy in elaborating protein structure (Saunders et al., 1957), histidine pK_a values [for a compilation, see Antosiewicz et al. (1996)], and protein folding pathways (Udgaonkar and Baldwin, 1988) was developed with RNase A. The ^{1}H-NMR resonances of the enzyme have been assigned, and the structure of the enzyme in solution has been determined (Rico et al., 1989; Robertson et al., 1989). This wealth of information has made RNase A an ideal model system for detailed biophysical analyses of protein structure–function relationships.

RNase A is a small protein (124 amino acid residues; 13.7 kDa) that catalyzes the hydrolysis of RNA in two distinct steps. In the first step, the side chain of His^{12} acts as a base to abstract a proton from the 2′-hydroxyl group of a substrate molecule and thereby facilitate attack on the phosphorus atom. The side chain of His^{119} acts as an acid to protonate the 5′-oxygen and facilitate its displacement (Findlay et al., 1961; Thompson and Raines, 1994). 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 (Cuchillo et al., 1993; Thompson et al., 1994).

RNase A may have converged upon a catalytic dyad that is similar to the catalytic triad of serine proteases and other hydrolases. Joint x-ray/neutron diffraction analysis indicates that His^{119} forms a hydrogen bond with Asp^{121} (Fig. 1). The importance of this hydrogen bond can be inferred from the conservation of His^{119} and Asp^{121} in all of the over 40 homologous pancreatic ribonucleases of known sequence (Beintema, 1987; Beintema et al., 1988).

We are interested in structure–function relationships that involve the His · · · Asp catalytic dyad of RNase A. Previously, we reported that replacing Asp^{121} 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 (Schultz et al., 1998). In addition, replacing Asp^{121} with an asparagine or alanine residue results in a loss of conformational stability at pH 6.0 of $\Delta\Delta G° = 2.0$ kcal/mol, from a total of 9.0 kcal/mol (Quirk et al., 1998). The magnitude of this loss is similar to that to transition state binding during catalysis.

Here, we have used site-directed mutagenesis and ^{1}H-NMR spectroscopy to gain additional insight into the role of Asp^{121} in the structure and function of RNase A. Specifically, we have determined how the pK_a of His^{119} changes when Asp^{121} is replaced with an asparagine or alanine residue. We have extended this analysis to the pK_a of His^{12} and the two other histidine residues in RNase A. Finally, we have assessed the effect of two different concentrations of the hydrolysis product 3′-UMP on the histidine pK_a values in all three enzymes.

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Abbreviations used: RNase A, bovine pancreatic ribonuclease A; *A*, absorbance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; 3′-UMP, uridine 3′-phosphate (otherwise Up).

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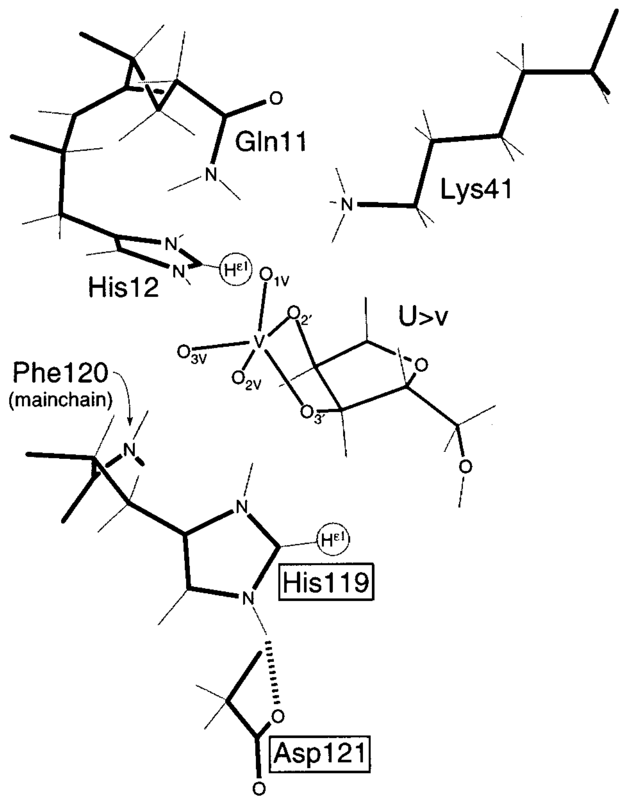

FIGURE 1 Crystalline structure of the active site of RNase A bound to uridine 2′,3′-cyclic vanadate (U > v), which is an analog of the transition state during catalysis. The structure was refined at 2.0 Å from x-ray and neutron diffraction data collected from crystals grown at pH 5.3 (Wlodawer et al., 1983). The structure has also been refined at 1.3 Å from x-ray diffraction data alone (Wladkowski et al., 1998). The His · · · Asp catalytic dyad is highlighted. The side chain of Phe^{120} and the uracil base are not shown.

MATERIALS AND METHODS

Materials

RNase A (Type XII-A, lyophilized, salt free) was from Sigma Chemical (St. Louis, MO). 3′-UMP (98% pure; Sigma Chemical) was used without further purification. D121N RNase A and D121A RNase A were produced in *Escherichia coli* and purified as described by Schultz et al. (1998). D_2O (99.9%), DCl solution (35% v/v in D_2O), and NaOD solution (40% w/v in D_2O) were from Isotec (Miamisburg, OH). DSS was from Cambridge Isotope Laboratories (Andover, MA).

Procedures

To facilitate the pH titrations by ^{1}H-NMR spectroscopy, exchangeable enzymic hydrogens were replaced with deuteriums. To exchange hydrogen for deuterium, lyophilized enzyme was dissolved in D_2O, lyophilized again, and redissolved in D_2O. DCl solution was used to adjust the pH* to 3.0, where pH* represents a direct reading of pH that is not corrected for a deuterium isotope effect. The resulting solution was heated to 60°C for 1 h to exchange the amide protons (Markley, 1975b). NaCl and DSS were then added to final concentrations of 0.20 M and 0.50 mM, respectively. 3′-UMP was added from a stock solution of 0.27 M.

^{1}H-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. Values of chemical shift (δ_{obs}) were recorded with respect to that of DSS. pH* was adjusted to values between 3.0 and 9.0 by adding commercial solutions of DCl or NaOD that had been diluted 10-fold with D_2O.

Both the deuterium exchange procedure and the pH titrations were performed in solutions with an enzyme concentration of 50 mg/mL. Enzyme concentrations were determined by assuming that $A = 0.72$ at 277.5 nm for a 1.0 mg/mL solution (Sela et al., 1957). 3′-UMP quantities were determined by weight.

Values of δ_{obs} for the imidazolyl C-2 proton (otherwise $H^{\epsilon 1}$; Fig. 1) at different values of $[H^+]$ were fitted to Eqs. 1, 2, or 3 (Fisher et al., 1998b):

$$\delta_{obs} = \delta_A \delta_{AH^+}\left(\frac{1 + K_{105}/[H^+]}{\delta_A + \delta_{AH^+}(K_{105}/[H^+])}\right) \quad (1)$$

$$\delta_{obs} = \delta_A \delta_{AH^+}\left(\frac{1 + \dfrac{K_{12a}K_{119a} + K_{119a}[H^+]}{[H^+](K_{12a} + [H^+])}}{\delta_A + \delta_{AH^+}\dfrac{K_{12a}K_{119a} + K_{119a}[H^+]}{[H^+](K_{12a} + [H^+])}}\right) \quad (2)$$

$$\delta_{obs} = \delta_A \delta_{AH^+}\left(\frac{1 + \dfrac{K_{12a}K_{119a} + K_{12a}[H^+]}{[H^+](K_{119a} + [H^+])}}{\delta_A + \delta_{AH^+}\dfrac{K_{12a}K_{119a} + K_{12a}[H^+]}{[H^+](K_{119a} + [H^+])}}\right) - \delta_o\left(\frac{\delta_o - \delta_{oH^+}}{\delta_o + \delta_{o^+}(K_o/[H^+])}\right) \quad (3)$$

Data for His^{105} were fitted to Eq. 1, which describes the pH titration of a group with one pK_a. The apparent interaction between the two active-site histidine residues—His^{12} and His^{119}—necessitates a fit involving the microscopic pK_a values of these two residues. Data for His^{119}, which has two microscopic pK_a values, were fitted to Eq. 2. Data for His^{12}, which has an acidic inflection in the pH* titration in addition to two microscopic pK_a values (Karpeisky and Yakovlev, 1981), were fitted to Eq. 3. The difference in the two microscopic pK_a values for each residue must be identical (Wyman and Gill, 1990). To accommodate this constraint, data for His^{12} and His^{119} were fitted simultaneously to Eqs. 2 and 3. All data were fitted by using the program MATHEMATICA 3.0 from Wolfram Research (Champaign, IL).

In Eqs. 2 and 3, an "a" subscript refers to the microscopic pK_a of one active-site histidine residue when the other active-site histidine residue is protonated, and a "b" subscript refers to the pK_a of this histidine when the other histidine residue is unprotonated. In Eq. 3, the "o" subscript refers to the pK_a for the acidic inflection observed in the His^{12} titration curve. Equations 1–3 are analogous to those used by Schechter and co-workers (Shrager et al., 1972) and by us (Chivers et al., 1997; Fisher et al., 1998b).

RESULTS

Titration of the C(2)-H chemical shifts of unliganded enzymes

RNase A has four histidine residues. The pK_a values for His^{12}, His^{119}, and His^{105} of RNase A can be determined by analyzing the chemical shift of the imidazolyl C-2 proton (Fig. 1) upon changing pH* (Markley, 1975b). The other histidine residue in RNase A, His^{48}, is inaccessible to solvent and its titration curve shows anomalous behavior with pH*, preventing a determination of its pK_a value (Markley, 1975a). Results from the pH titration of the unliganded ribonucleases are shown in Fig. 2, and the calculated pK_a values and limiting chemical shifts are listed in Table 1. The

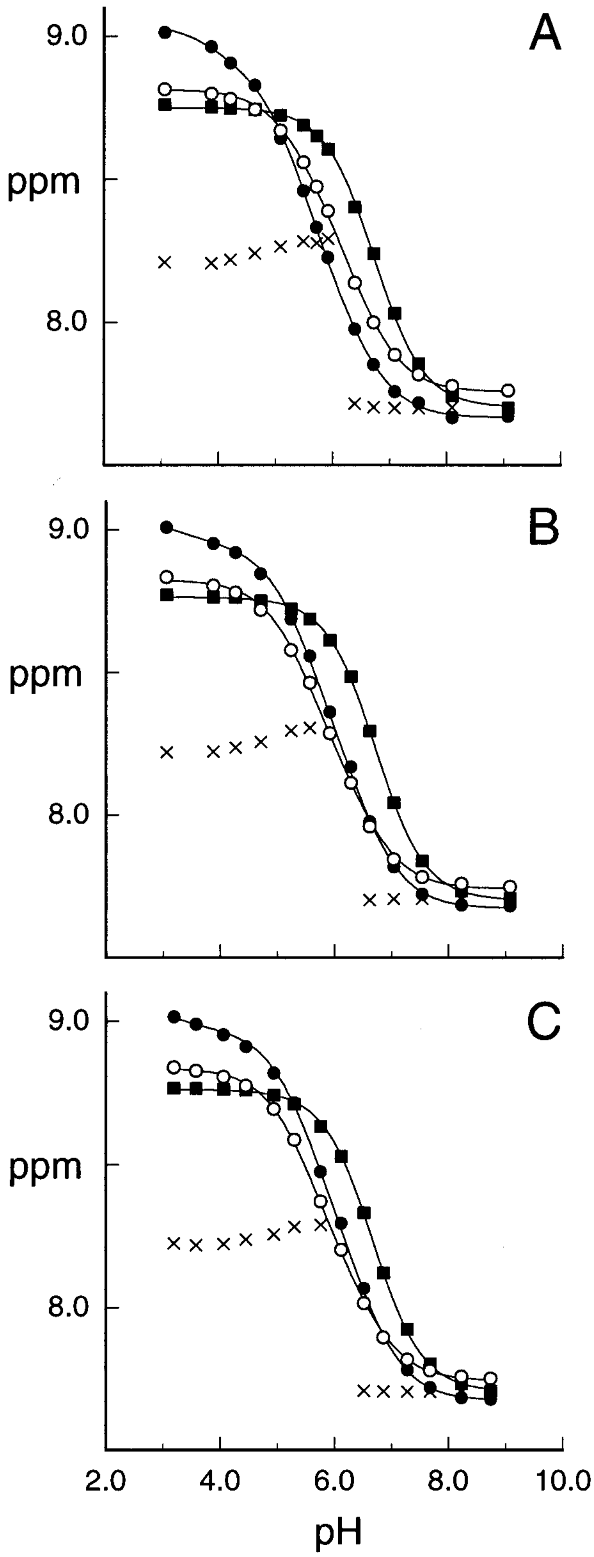

FIGURE 2 pH dependence of the histidine $^1H^{\epsilon 1}$ signals of wild-type ribonuclease A and the D121N and D121A variants in D_2O. (*A*) Wild-type ribonuclease A (*B*) D121N ribonuclease A. (*C*) D121A ribonuclease A. Chemical shifts are shown for all four histidine residues: His^{12} (●), His^{119} (○), His^{105} (■), and His^{48} (×). Titrations were carried out at 25°C in buffer containing enzyme (3.5 mM) and NaCl (0.20 M). The pK_a values determined from fitting the data to Eqs. 1–3 are listed in Table 1.

pK_a values of His^{12}, His^{119}, and His^{105} in wild-type RNase A and the D121N and D121A variants were well defined; the largest contribution to error appears to lie in the measurement of pH itself. Such systematic errors largely disappear when differences in pK_a values are calculated for any given titration.

Lys^{41}, like His^{12} and His^{119}, resides in the active site of RNase A. The pK_a of the side chain of Lys^{41} is near 9.0 (Jentoft et al., 1981), a value much greater than that of any histidine residue in RNase A. Accordingly, a perturbation to the pK_a of Lys^{41} upon replacing Asp^{121} is unlikely to have a substantial effect on the titrations and interpretations reported herein.

The microscopic pK_a values indicate that protonation of the active-site histidine residues has negative cooperatively (that is, protonation of one histidine residue disfavors protonation of the other) as had been observed previously (Markley and Finkenstadt, 1975). The negative cooperatively observed here is slightly greater than that in the previous study, perhaps because the previous observations were made in a solution of higher salt concentration (0.30 M versus 0.20 M NaCl), which should reduce Coulombic interactions.

Replacing Asp^{121} with an asparagine or alanine residue decreases the pK_a of His^{119} by 0.11 units (which is the average of the microscopic values), and increases the pK_a of His^{12} by ~0.15 units. For all three enzymes, the titration of His^{12} displayed an inflection in the acidic region (Karpeisky and Yakovlev, 1981). The proximity of this inflection to the pK_a of His^{12} and the lack of data below pH* 3 made for a large error in its determination. For all three enzymes, the titration of His^{48} also displayed an inflection in the acidic region, as had been seen previously (Markley, 1975b). The effect of the substitutions on the titration of His^{48} was minor. No effect was observed on the titration of His^{105}. The limiting chemical shifts of each histidine residue were almost identical for all three enzymes.

Titration of the C(2)-H chemical shifts of enzyme · product complexes

pH titrations of wild-type RNase A and the D121N and D121A variants were performed in the presence of the reaction product 3′-UMP. In these titrations, the concentration of enzyme was 3.5 mM and that of 3′-UMP was either 1.5 mM or 11 mM. The results are shown in Figs. 3 and 4, and the calculated pK_a values and limiting chemical shifts are listed in Table 1. The data were fitted without regard to the association of 3′-UMP.

In the wild-type enzyme, His^{119} is more perturbed by the presence of 3′-UMP than is His^{12}, as had been observed previously (Eftink and Biltonen, 1983). In the presence of 11 mM 3′-UMP, replacing Asp^{121} with an asparagine residue reduces the pK_a of His^{119} by 0.36 and increases that of His^{12} by 0.21. Replacing Asp^{121} with an alanine reduces the pK_a of His^{119} by 0.97 and increases that of His^{12} by 0.35.

TABLE 1 Microscopic pK_a values and limiting C(2)-H chemical shifts of histidine residues in wild-type ribonuclease A and the D121N and D121A variants in the absence and presence of uridine 3′-phosphate

Residue	[3′-UMP] (mM)	Wild-Type Ribonuclease A			D121N Ribonuclease A			D121A Ribonuclease A		
		pK_a*	δ_{low}	δ_{high}#	pK_a	δ_{low}	δ_{high}	pK_a	δ_{low}	δ_{high}
His[12]	0	5.74 ± 0.03	8.89 ± 0.03	7.67 ± 0.01	5.84 ± 0.02	8.96 ± 0.02	7.67 ± 0.01	5.88 ± 0.02	8.98 ± 0.01	7.67 ± 0.01
		6.12 ± 0.04			6.28 ± 0.06			6.32 ± 0.04		
		3.99 ± 0.28	9.01 ± 0.03	8.86	3.19 ± 1.35	8.96 ± 0.11	8.86	2.92 ± 1.87	8.97 ± 0.31	8.86
His[119]	0	5.94 ± 0.02	8.82 ± 0.01	7.76 ± 0.01	5.80 ± 0.03	8.82 ± 0.01	7.74 ± 0.01	5.80 ± 0.02	8.84 ± 0.01	7.74 ± 0.00
		6.32 ± 0.03			6.24 ± 0.04			6.24 ± 0.03		
His[105]	0	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
His[12]	1.5	6.33 ± 0.02	8.75 ± 0.01	7.66 ± 0.01	6.27 ± 0.02	8.78 ± 0.02	7.68 ± 0.05	6.39 ± 0.01	8.86 ± 0.01	7.68 ± 0.00
		6.62 ± 0.07			6.49 ± 0.06			6.47 ± 0.06		
		4.14 ± 0.26	8.98 ± 0.02	8.86	4.26 ± 0.15	9.05 ± 0.02	8.86	3.78 ± 0.15	9.02 ± 0.02	8.86
His[119]	1.5	6.74 ± 0.06	8.75 ± 0.02	7.78 ± 0.01	6.73 ± 0.06	8.67 ± 0.02	7.74 ± 0.01	6.37 ± 0.04	8.73 ± 0.01	7.75 ± 0.01
		7.03 ± 0.02	8.91 ± 0.02	8.86	6.95 ± 0.02	8.97 ± 0.02	8.86	6.44 ± 0.03	9.00 ± 0.02	8.86
		4.34 ± 0.59			4.63 ± 0.18			3.89 ± 0.26		
His[105]	1.5	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
His[12]	11	6.45 ± 0.03	8.72 ± 0.02	7.69 ± 0.01	6.66 ± 0.01	8.75 ± 0.04	7.71 ± 0.00	6.80 ± 0.01	8.78 ± 0.00	7.71 ± 0.00
		6.35 ± 0.24			6.30 ± 0.13			6.50 ± 0.05		
		3.77 ± 0.36	8.99 ± 0.02	8.86	2.63 ± 1.01	9.19 ± 1.53	8.86	2.78 ± 0.25	9.26 ± 0.15	8.86
His[119]	11	7.95 ± 0.29	8.69 ± 0.03	7.76 ± 0.03	7.86 ± 0.15	8.65 ± 0.01	7.74 ± 0.01	7.18 ± 0.05	8.64 ± 0.01	7.75 ± 0.00
		7.85 ± 0.07	8.96 ± 0.04	8.86	7.49 ± 0.02	9.04 ± 0.02	8.86	6.88 ± 0.02	9.09 ± 0.01	8.86
		4.06 ± 0.75			3.88 ± 0.13			3.89 ± 0.08		
His[105]	11	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

Data were obtained at 25°C in buffers containing 3′-UMP (0, 1.5 mM, or 11 mM) and NaCl (0.20 M). Values were determined by fitting the experimental data in Figs. 2–4 to Eq. 1 for His[105], Eq. 2 for His[119], and Eq. 3 for His[12]. Errors were determined by a nonlinear least-squares fit of the data to Eqs. 1–3.
*For His[12] and His[119], the first pK_a value is that observed when the other active-site histidine residue is protonated (corresponding to K_{12a} and K_{119a} in Eqs. 2–4); the second, when it is unprotonated (K_{12b} and K_{119b}).
#The δ_{high} values for additional pK_a values are defined to be 8.86.

These comparisons are based upon the microscopic pK_a values of His[119] (in which His[12] is not protonated) and the microscopic pK_a values of His[12] (in which His[119] is protonated), as this combination is the predominant one. The presence of 3′-UMP produces a new pK_a of 3.9 during the titration of His[119]. The effect of 3′-UMP on the cooperativity of the titrations is discussed below.

In the course of titrating D121N RNase A in the presence of 3′-UMP, we observed that the chemical shift of the C-4 proton (otherwise $H^{\delta 2}$) of His[119] had shifted downfield at acidic pH by ~0.3 ppm, to a spectral region in which it is no longer obscured by signals from phenylalanine and tyrosine residues. A comparison of spectra of D121N RNase A with that for the wild-type enzyme led us to conclude that our assignment of the C-4 proton of His[119] and that of Kaptein and co-workers are correct (Lenstra et al., 1979).

DISCUSSION

Unliganded enzymes

We find that upon loss of Asp[121], the pK_a of His[119] drops by only 0.1 unit: a value much smaller than that expected for the loss of a hydrogen bond between functional groups of opposite charge (Fersht et al., 1985). Why is the pK_a of His[119] not perturbed more by Asp[121]? The side chain of His[119] is flexible, occupying two distinct positions in wild-type RNase A (Borkakoti et al., 1982). In position A, $N^{\epsilon 2}$ of His[119] forms a hydrogen bond with $O^{\delta 1}$ of Asp[121] (Fig. 1), with the side chain of His[119] having torsion angles of $\chi_1 = 149°$ and $\chi_2 = -101°$. In position B, the imidazolyl group is removed from Asp[121] by 7 Å, with $\chi_1 = -69°$ and $\chi_2 = -63°$. In analyses of the crystalline and solution structure of RNase A, His[119] has been found in position A, position B, or both [for reviews, see Gilliland (1997) and González et al. (1997)]. The pH-dependencies of the thermodynamic stabilities of wild-type RNase A and the D121N, D121A, and H119A variants suggest that His[119] of the wild-type enzyme resides predominantly in position A when its side chain is protonated (Quirk, 1996; Quirk et al., 1998). These studies also revealed that the pK_a of Asp[121] is perturbed by one unit upon the folding of RNase A. One possibility, then, is that the deprotonated state of His[119] favors position B, which is removed from Asp[121]. A cation (e.g., Na^+) could then replace the neutral His[119] side chain in position A. A second possibility is that another residue buffers the effect of removing Asp[121]. This residue could be Glu[111], which is only 5 Å from the imidazolyl group of His[119] when it resides in position B.

Replacing Asp[121] with an asparagine or alanine residue increases the pK_a of His[12] by 0.15, an effect larger and in the opposite direction to that on His[119]. A simple Coulombic calculation indicates that removing the anionic side chain of Asp[121] is expected to *decrease* the pK_a of His[12] by ~0.2 (= $0.4 \times 7^2/10^2$ by Coulomb's law). This calculation is based on the observations that protonating His[12] decreases the pK_a of His[119] by 0.4 units (and vice versa) and that His[12] is

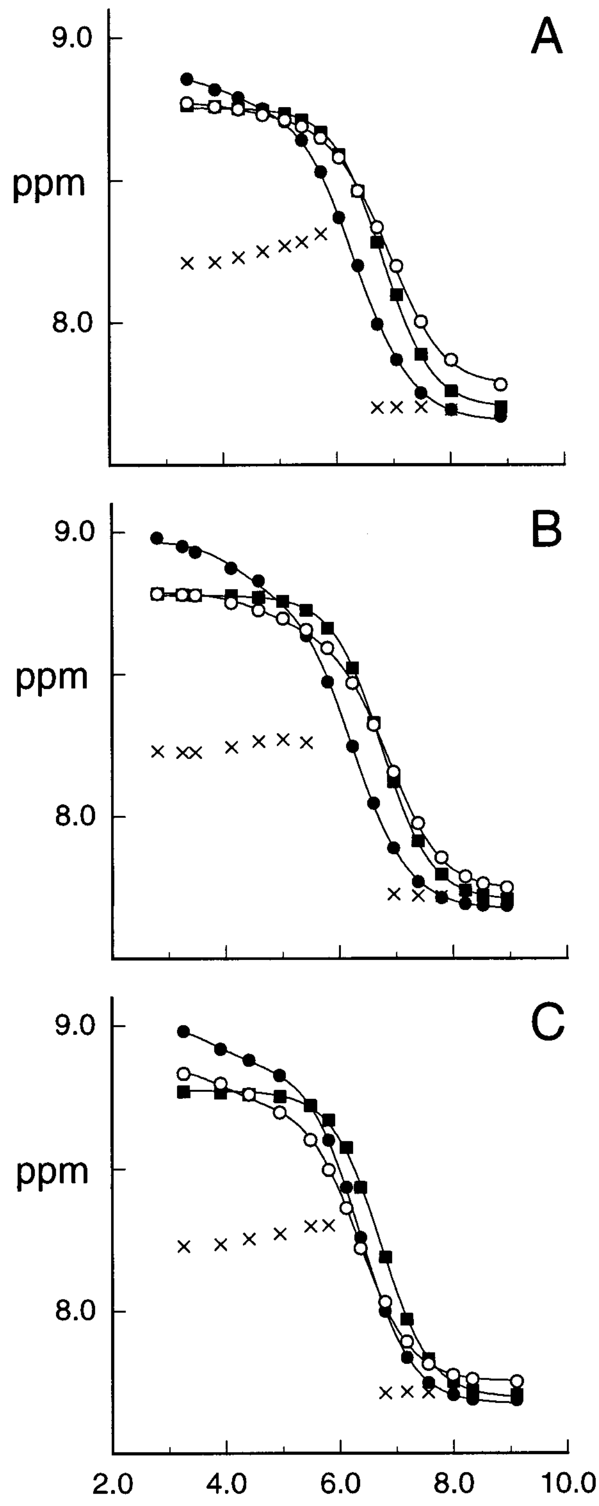

FIGURE 3 pH dependence of the histidine $^1H^{\epsilon1}$ signals of wild-type ribonuclease A and the D121N and D121A variants in D_2O containing a low concentration of 3′-UMP. (*A*) Wild-type ribonuclease A. (*B*) D121N ribonuclease A. (*C*) D121A ribonuclease A. Chemical shifts are shown for all four histidine residues: His^{12} (●), His^{119} (○), His^{105} (■), and His^{48} (×). Titrations were carried out at 25°C in buffer containing enzyme (3.5 mM), 3′-UMP (1.5 mM), and NaCl (0.20 M). The pK_a values determined from fitting the data to Eqs. 1–3 are listed in Table 1.

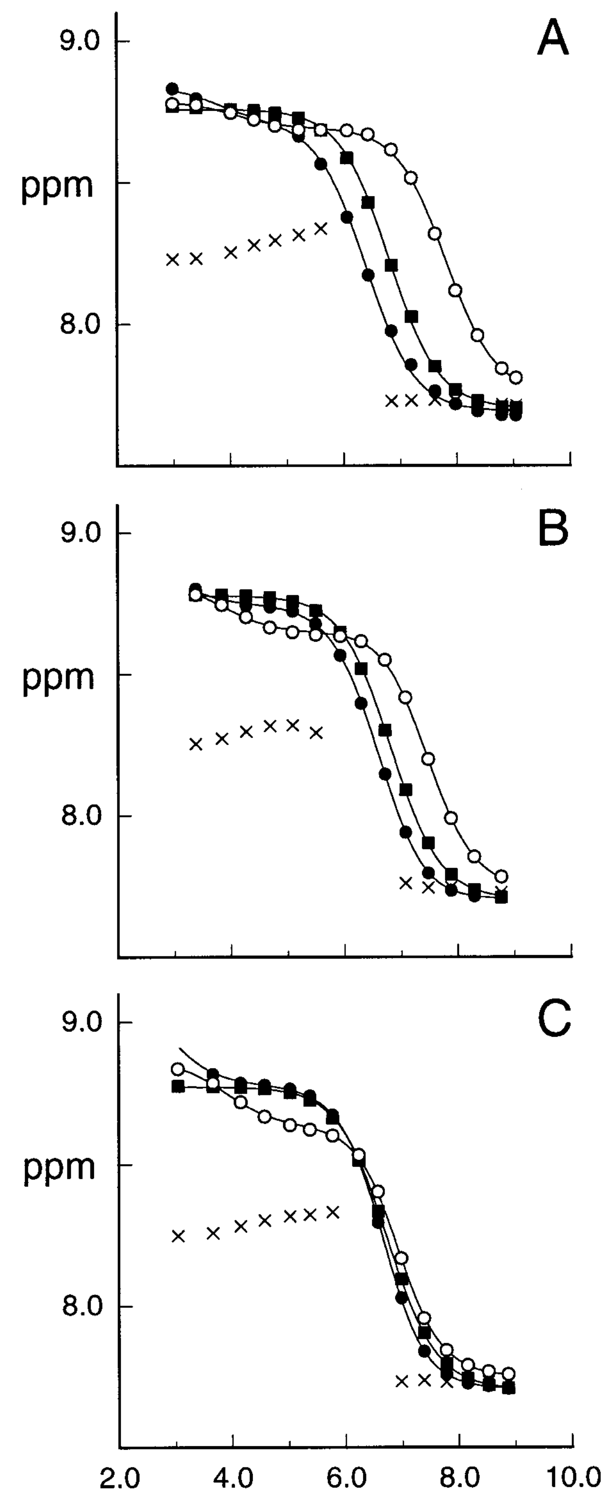

FIGURE 4 pH dependence of the histidine $^1H^{\epsilon1}$ signals of wild-type ribonuclease A and the D121N and D121A variants in D_2O containing a high concentration of 3′-UMP. (*A*) Wild-type ribonuclease A. (*B*) D121N ribonuclease A. (*C*) D121A ribonuclease A. Chemical shifts are shown for all four histidine residues: His^{12} (●), His^{119} (○), His^{105} (■), and His^{48} (×). Titrations were carried out at 25°C in buffer containing enzyme (3.5 mM), 3′-UMP (11 mM), and NaCl (0.20 M). The pK_a values determined from fitting the data to Eqs. 1–3 are listed in Table 1.

separated from His^{119} and Asp^{121} by 7 and 10 Å, respectively, and on the assumption that the dielectric constant is similar for both interactions. A role for His^{119}—through its conformational flexibility—in this curious perturbation of His^{12} is difficult to justify because the His^{12}–His^{119} distance is similar in the A and B positions, and the change in the microscopic pK_a values is similar when His^{119} is in the A or B position. The replacement of Asp^{121} therefore appears to have effects on a more global scale that quite possibly involves some of the many basic residues in RNase A.

Our results conflict with those from a study that used a semisynthetic RNase A (Cederholm et al., 1991). That enzyme, RNase-(1–118) · (111–124), consists 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 Asp^{121} of the semisynthetic enzyme with an asparagine residue increases the macroscopic pK_a of His^{119} by 0.05 and decreases the macroscopic pK_a of His^{12} by 0.09; effects that are in opposition to ours [Cederholm et al. (1991) did not report any microscopic pK_a values]. Most notably, the difference between the pK_a values of His^{119} and His^{12} ($\Delta pK_a = pK_a^{His119} - pK_a^{His12}$) increases from 0.3 for the parent semisynthetic enzyme to 0.45 for the D121N variant, but ΔpK_a decreases from 0.18 for authentic wild-type RNase A to −0.04 for D121N RNase A. This deviation is consistent with the semisynthesis having introduced unpredictable structural perturbations. Indeed, x-ray diffractions analyses of the two semisynthetic enzymes revealed many small changes in their structures (de Mel et al., 1992). In contrast, the structure of D121N RNase A is essentially identical to that of the authentic wild-type enzyme (Schultz et al., 1998).

Ribonuclease A · 3′-UMP complexes

Both NMR spectroscopy and x-ray diffraction analysis have been used to determine the three-dimensional structures of complexes of RNase A with various mononucleotides, including 3′-UMP (Bruix et al., 1991). These structures have been the subject of recent reviews (Gilliland, 1997; González et al., 1997). No significant perturbation to enzymic structure is observed upon formation of the complexes, which have active sites analogous to that shown in Fig. 1.

Our pH titrations reveal new insight on the interaction between RNase A and 3′-UMP. The relationship between the microscopic pK_a values of the active-site histidine residues can be defined by the parameter c, with $c > 1$ indicating positive cooperativity and $c < 1$ indicating negative cooperativity (Wyman and Gill, 1990). For titrations in the active site of RNase A, $c = K_{12b}/K_{12a} = K_{119b}/K_{119a}$ (Fisher et al., 1998b). The values for c for wild-type RNase A and the D121N and D121A variants in the absence and presence of 3′-UMP are listed in Table 2. In the absence of 3′-UMP, $c < 1$ for all three enzymes. The presence of 3′-UMP increases the value of c. Moreover, in the presence of a high concentration of 3′-UMP, $c > 1$ for all three enzymes. In other words, protonation of one active-site histidine residue then *favors* protonation of the other. Such positive cooperativity is unusual for the titration of two proximal acids of like charge. This result is consistent with the anionic phosphoryl group of 3′-UMP binding more strongly to the active site when both histidine residues are protonated and thus cationic.

TABLE 2 Cooperativity (*c*) values from the analysis of the microscopic pK_a values of the active-site histidine residues of wild-type ribonuclease A and the D121N and D121A variants in the absence and presence of uridine 3′-phosphate

[3′-UMP] (mM)	Ribonuclease A		
	Wild-Type	D121N	D121A
0	0.42	0.36	0.36
1.5	0.51	0.60	0.84
11	1.3	2.3	2.0

Values were calculated from the data in Table 1 and the equation: $c = [(K_{12b}/K_{12a}) + (K_{119b}/K_{119a})]/2$.

To extract as much information as possible from the ^{1}H-NMR data, we attempted to fit the microscopic pK_a values in Table 1 to Eq. 4, which describes the pH-dependence of the dissociation constant (K_d) of the enzyme · 3′-UMP complex:

$$K_d = \frac{K_d^{int}\left(\frac{K_{12b}K_{119a}}{K_{12b}^I K_{119a}^I}\right)}{1 + \frac{[H^+]}{K_{119a}^I} + \frac{[H^+]K_{119b}^I + [H^+]^2}{K_{12b}^I K_{119a}^I} + \frac{[H^+]^3}{K_{12b}^I K_{119a}^I K_p^I}} + \frac{K_d^{int}\left(\frac{K_{12b}}{K_{12b}^I}\right)}{1 + \frac{K_{119a}^I}{[H^+]} + \frac{K_{119b}^I + [H^+]}{K_{12b}^I} + \frac{[H^+]^2}{K_{12b}^I K_p^I}} + \frac{K_d^{int}\left(\frac{K_{119b}}{K_{119b}^I}\right)}{1 + \frac{K_{12b}^I + [H^+]}{K_{119b}^I} + \frac{[H^+]^2}{K_{119b}^I K_p^I} + \frac{K_{12b}^I K_{119a}^I}{K_{119b}^I [H^+]}} + \frac{K_d^{int}}{1 + \frac{[H^+]}{K_p^I} + \frac{K_{12b}^I K_{119a}^I + K_{12b}^I K_{119b}^I}{[H^+]^2}} + \frac{K_d^{int}\left(\frac{K_p^I}{K_p}\right)}{1 + \frac{K_p^I}{[H^+]} + \frac{K_{119b}^I K_p^I}{[H^+]^2} + \frac{K_{12b}^I K_{119a}^I K_p^I}{[H^+]^3}} \quad (4)$$

where K_{12a} refers to His^{12} when His^{119} is protonated, K_{119a} refers to His^{119} when His^{12} is protonated, K_{12b} refers to His^{12} when His^{119} is not protonated; K_{119b} refers to His^{119} when His^{12} is not protonated, and K_p refers to the phosphoryl group of 3′-UMP as obtained from steady-state kinetic analyses (Schultz et al., 1998). The K_d^{int} is the intrinsic dissociation constant of the enzyme · 3′-UMP complex when the enzymic active site is fully protonated and the inhibitor is fully deprotonated (Fig. 5). Terms containing an "I" superscript refer to pK_a values in the presence of 3′-UMP. The equilibrium constants in Eq. 4 are related by the thermodynamic cube shown in Fig. 5. The concentration of enzyme · 3′-UMP complex ([E · I]) at any pH can be determined from the values of K_d and the equation:

$$K_d = \frac{([E]_{total} - [E \cdot I])([I]_{total} + [E \cdot I])}{[E \cdot I]} \quad (5)$$

where $[E]_{total}$ is the total concentration of RNase A and $[I]_{total}$ is the total concentration of 3′-UMP. The calculated chemical shift (δ_{calc}) is then simply the weighted sum of the chemical shifts for the unliganded (δ_{free}) and bound (δ_{bound}) enzyme, as in the equation:

$$\delta_{calc} = \delta_{free}\left(1 - \frac{[E \cdot I]}{[E]_{total}}\right) + \delta_{bound}\frac{[E \cdot I]}{[E]_{total}} \quad (6)$$

Favorable Coulombic interactions contribute to the affinity of RNase A for 3′-UMP. Isothermal titration calorimetry indicates that the RNase A · 3′-UMP complex has K_d = 0.054 mM at 25°C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M) (Fisher et al., 1998b). This solution has a $[Na^+]$ of 0.142 M. Because the pH titrations described herein were performed in solutions with a $[Na^+]$ of 0.20 M, the RNase A · 3′-UMP complex is likely to have $K_d > 0.054$ M. To attempt to fit the microscopic pK_a values to Eqs. 4–6, we assumed that only the active site is occupied when 3′-UMP is present at a low concentration, such as 1.5 mM.

Our attempts to fit the experimental data to Eqs. 4–6 revealed a problem. Chemical shifts observed in the presence of 1.5 mM 3′-UMP should fall between that of the unliganded enzyme and that in the presence of 11 mM 3′-UMP. Yet, the chemical shifts calculated with Eq. 6 do not obey this constraint. The perturbation to these chemical shifts by a low concentration of 3′-UMP was equal to or greater than that from a high concentration. We conclude that the RNase A plus 3′-UMP system is at least a three-body one when 3′-UMP is present at high concentration. A second molecule of RNase A would have resulted in significant spectral 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. Our conclusion is in accord with kinetic data suggesting that more than one molecule of cytidine 2′,3′-cyclic phosphate (C > p) binds to RNase A when [C > p] > 10 mM (Moussaoui et al., 1998).

Evidence for subsites in RNase A comes from many workers [for reviews, see Parés et al. (1991); Nogués et al. (1995); Raines (1998)]. That RNase A binds and cleaves polymeric substrates is itself suggestive of enzymic subsites that bind to monomeric units. A number of x-ray diffraction analyses on complexes with substrate analogs have revealed the presence of subsites (Eftink and Biltonen, 1983; McPherson et al., 1986; Fontecilla-Camps et al., 1994). In addition, Irie and co-workers used ^{31}P-NMR spectroscopy to provide evidence of binding sites for three phosphoryl groups and two bases (Irie et al., 1984). Recently, a fourth phosphoryl-group subsite has been discovered (Fisher et al., 1998a), and the existence of a fifth nucleotide subsite has been inferred (Moussaoui et al., 1998). Perhaps most germane to this work is the finding that adenine, adenosine, adenosine 3′-phosphate, and adenosine 5′-phosphate increase the rate of the RNase A-catalyzed hydrolysis of C > p (Wieker and Witzel, 1967; Haffner and Wang, 1973; Moussaoui et al., 1998).

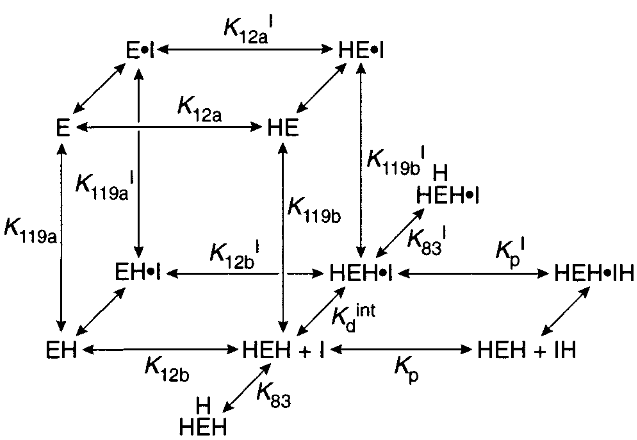

FIGURE 5 Thermodynamic cube showing the relationships between the titration of the imidazolyl groups of His^{12} and His^{119} of ribonuclease A (E), the phosphoryl group of 3′-UMP (I), and a residue that could be Asp^{83}, in both the unliganded enzyme and the enzyme · 3′-UMP complex. The nomenclature used is as in Eq. 4.

Acidic inflections

The inflection with a pK_a of 3.9 observed in the titration of His^{119} in the presence of 3′-UMP almost certainly arises from the deprotonation of the phosphoryl group. The decrease from a pK_a of 5.8 for free 3′-UMP is consistent with its interaction with the two active-site histidine residues (Tanokura, 1983). A thermodynamic determination of K_p (Flogel and Biltonen, 1975) as a function of pH supports this view, as do studies that relied on ^{31}P-NMR spectroscopy (Gorenstein and Wyrwicz, 1973).

The inflection seen in the titration of His^{12} has been reported widely, but its assignment less so. Perhaps the most thoughtful analysis of the titration behavior of His^{12} comes from Karpeisky and Yakovlev (1981). These workers concluded from much evidence that the functional group responsible for the inflection is the side chain of Asp^{83}, which mediates its effect through Thr^{45} and its interaction with both the uracil base and His^{12}. The importance of hydrogen bonding between Thr^{45} and Asp^{83} to the binding of a uracil base has been demonstrated by site-directed mutagenesis (delCardayré and Raines, 1995).

Low-barrier hydrogen bonds?

The small contribution of Asp[121] to catalysis belies the importance of the hydrogen bond between His[119] and Asp[121] (Schultz et al., 1998). Nonetheless, a similar His ··· Asp dyad has been proposed to form a low-barrier hydrogen bond of extraordinary strength during catalysis by the protease chymotrypsin (Frey et al., 1994; Cassidy et al., 1997), though this interpretation is controversial (Ash et al., 1997). One criterion for such a bond is an ^{1}H chemical shift of 17–20 ppm (Cleland and Kreevoy, 1994). The ^{1}H chemical shift of $N_{\epsilon 2}$H of His[119] appears at a much higher field, <12 ppm (J. L. Markley, personal communication). By this criterion, the His ··· Asp dyad of RNase A does not have a low-barrier hydrogen bond. In theory, the His ··· Asn dyad of D121N RNase A could also form a low-barrier hydrogen bond. Another criterion for such a bond is that it arise from acids with matched pK_a values (Cleland, 1992). The pK_a values of imidazole [14.2 (Walba and Isensee, 1955)] and acetamide [15.1 (Bordwell, 1988)], which are reasonable models for the side chains of His[119] and Asn[121], respectively, are indeed similar. The existence of a low-barrier hydrogen bond would, however, require that the side chain of His[119] be in its neutral imidazole form. ^{1}H-NMR titrations of His[119] in the D121N variant (Figs. 2–4) and pH–rate profiles for catalysis by D121N RNase A (Schultz et al., 1998) indicate that His[119] titrates between its imidazole and imidazolium forms with a pK_a value similar to that of His[119] in wild-type RNase A. These data deny the existence of a low-barrier hydrogen bond in the His ··· Asn dyad.

CONCLUSIONS

Though conserved during evolution, Asp[121] of RNase A has little effect on the ionization of His[119]. ^{1}H-NMR spectroscopy has shown that neither the microscopic pK_a values of His[119] nor those of any other histidine residue is perturbed by more than 0.2 units when Asp[121] is replaced by an asparagine or alanine residue. Microscopic pK_a values of His[12] and His[119] in the presence of 3′-UMP exhibit positive rather than negative cooperativity. Finally, ^{1}H-NMR data provide evidence that RNase A can bind a second molecule of the 3′-UMP product within its active site. As in previous studies (Quirk et al., 1998; Schultz et al., 1998), we conclude that the mere presence of a His ··· Asp catalytic dyad in an active site is not a mandate for its playing a crucial role in the structure or function of the enzyme.

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