Published in final edited form as:

Biochemistry. 1998 June 23; 37(25): 8886–8898. doi:10.1021/bi972766q.

His...Asp Catalytic Dyad of Ribonuclease A: Structure and Function of the Wild-Type, D121N, and D121A Enzymes†

L. Wayne Schultz, David J. Quirk[‡], and Ronald T. Raines^{*}

Departments of Biochemistry and Chemistry, University of Wisconsin – Madison, Madison, WI 53706

Abstract

The sidechains of histidine and aspartate residues form a hydrogen bond in the active sites of many enzymes. In serine proteases, the His...Asp hydrogen bond of the catalytic triad is known to contribute greatly to catalysis, perhaps via the formation of a low-barrier hydrogen bond. In bovine pancreatic ribonuclease A (RNase A), the His...Asp dyad is composed of His119 and Asp121. Previously, site-directed mutagenesis was used to show that His119 has a fundamental role—to act as an acid during catalysis of RNA cleavage [Thompson & Raines (1994), *J. Am. Chem. Soc.* 116, 5467–5468]. Here, Asp121 was replaced with an asparagine or alanine residue. The crystalline structures of the two variants were determined by X-ray diffraction analysis to a resolution of 1.6 Å with an *R*-factor of 0.18. Replacing Asp121 with an asparagine or alanine residue does not perturb the overall conformation of the enzyme. In the structure of D121N RNase A, N_δ rather than O_δ of Asn121 faces His119. This alignment in the crystalline state is unlikely to exist in solution because catalysis by the D121N variant is not compromised severely. The steady-state kinetic parameters for catalysis by the wild-type and variant enzymes were determined for the cleavage of uridylyl(3'→5')adenosine and poly(cytidylic acid), and for the hydrolysis of uridine 2',3'-cyclic phosphate. Replacing Asp121 decreases the values of k_{cat}/K_m and k_{cat} for cleavage by 101-fold (D121N) and 10²-fold (D121A). Replacing Asp121 also decreases the values of k_{cat}/K_m and k_{cat} for hydrolysis by 10^{0.5}-fold (D121N) and 10-fold (D121A), but has no other effect on the pH-rate profiles for hydrolysis. There is no evidence for the formation of a low-barrier hydrogen bond between His119 and either an aspartate or an asparagine residue at position 121. Apparently, the major role of Asp121 is to orient the proper tautomer of His119 for catalysis. Thus, the mere presence of a His...Asp dyad in an enzymic active site is not a mandate for its being crucial in effecting catalysis.

Keywords

catalytic dyad; catalytic triad; low-barrier hydrogen bond; pH-rate profile; ribonuclease A; serine protease; site-directed mutagenesis; X-ray crystallography

The amino acid motifs available to enzymic catalysts are diverse. Still, many enzymes fall into classes wherein great similarities exist. One well-studied class is that of the serine proteases (1). This class of enzymes has an active-site motif known as the catalytic triad, which is composed of the residues Ser...His...Asp linked by hydrogen bonds (2,3).

[†]This work was supported by Grant GM44783 (NIH). X-ray data collection and computational facilities were supported by Grant BIR-9317398 (NSF). L.W.S. was supported by postdoctoral fellowship CA69750 (NIH). D.J.Q. was supported by Cellular and Molecular Biology Training Grant GM07215 (NIH).

^{*}Author to whom correspondence should be addressed.

[‡]Present address: School of Pharmacy, University of Wisconsin – Madison, Madison, WI 53706.

The importance ascribed to the Ser···His···Asp motif is in large part due to its presence in the active sites of nonhomologous serine proteases (4). In recent years, the triad has also been found in several lipases (5,6), *Fusarium solani* cutinase (7), and the Sindbis virus core protein (8). Replacing the aspartate residue in the catalytic triad of human lipoprotein lipase with a glycine is responsible for familial Type I hyperlipoproteinemia (9).

Similar to the catalytic triad, a His···Asp motif is found in the active sites of serine carboxypeptidase (10), acetylcholinesterase (11), phospholipase A₂ (12), haloalkane dehalogenase (13), diene lactone hydrolase (14), a variety of zinc-dependent enzymes (15), and pancreatic ribonucleases (16,17). This His···Asp motif is known as the “catalytic dyad”. With the exception of the pancreatic ribonucleases, enzymes containing the His···Asp dyad have *syn*-oriented carboxylates (18), though the benefit to catalysis of a *syn* vs *anti* orientation appears to be minimal (19,20).

In all enzymes containing a catalytic triad or dyad, an hydroxyl group functions as a nucleophile. The role of the histidine residue is to abstract a proton from the hydroxyl group and thereby to increase the nucleophilicity of the oxygen. The role of the aspartate residue has been the subject of much study and debate, as has the importance of the catalytic triad in general (21).

The catalytic triad was once thought to act in a “charge relay mechanism”. Specifically, it was postulated that a proton was removed from the serine residue by histidine while another proton was being removed from histidine by aspartate (2,22). The unperturbed pK_a of aspartic acid is too low to favor such a mechanism, and the general consensus today is that the charge relay mechanism is not operative. Instead, the aspartate residue forms a hydrogen bond that could serve both to orient the histidine sidechain and to increase its pK_a, enhancing its ability to abstract a proton from the serine residue.

Site-directed mutagenesis has been used to illuminate the role and importance of the aspartate residue in the catalytic triad of two serine proteases. Replacing the aspartate residue with asparagine in trypsin (23,24) or alanine in subtilisin (25) leads to a 10⁴-fold reduction in catalytic activity. This decrease suggests that the aspartate residue plays a critical role in catalysis. X-ray diffraction analysis of the trypsin variant revealed, however, that the catalytic histidine residue was being stabilized as an unproductive tautomer by its interaction with asparagine (26). This result suggests that one role of the aspartate residue in a Ser···His···Asp triad is to orient the proper tautomer of histidine.

Two attempts have thus far been made to determine the role of Asp121, the aspartate in the catalytic dyad of bovine pancreatic ribonuclease A [RNase A¹; E.C. 3.1.27.5; (27)]. In one study, Asp121 was replaced with asparagine in a semisynthetic enzyme (28). This semisynthetic RNase A, RNase(1 – 118)•(111 – 124), consists of a noncovalent complex between residues 1 – 118 of RNase A (obtained from proteolytic digestion of RNase A), and an overlapping synthetic peptide composed of the fourteen C-terminal residues of RNase A, except with Asp121 replaced by an asparagine residue. The semisynthetic D121N analog has 5% of the catalytic activity of the analogous wild-type semisynthetic enzyme. A difficulty with interpreting the results of this study, however, is that the three-dimensional structure of the semisynthetic D121N analog exhibits numerous changes compared to that of the analogous wild-type semisynthetic enzyme (29,30). In another study, site-directed mutagenesis was used to replace Asp121 in RNase A itself with a glutamate residue (31). The D121E enzyme has 17% of the activity of the wild-type enzyme for the hydrolysis of 0.1 mM C>p and 16% for the hydrolysis of 0.4 mM C>p. No other characterization of this variant has been reported. In related work, replacing Asp116 of the His···Asp dyad of angiogenin, a homolog of RNase A,

with an asparagine or alanine residue was shown to *increase* ribonucleolytic activity by 8- and 15-fold, respectively (32).

Here, we address the role of the aspartate residue in the catalytic dyad of RNase A. RNase A catalyzes the two-step hydrolysis of the P–O^{5'} bond of RNA on the 3' side of pyrimidine residues. Figure 1 depicts the classical mechanism for these reactions (33). In the transphosphorylation step, His119 of the His···Asp dyad acts as an acid. The slow hydrolysis of the 2',3'-cyclic phosphate occurs separately and resembles the reverse of transphosphorylation (34,35). It is in this second step that the action of the catalytic dyad of RNase A most resembles that of the catalytic triad. The amino acid sequences of pancreatic ribonucleases from over forty vertebrates are known (16,17), and are evolving rapidly (36). The catalytic dyad is conserved in each of these ribonucleases, suggesting that these residues are important. We have created RNase A variants in which Asp121 is replaced with an

¹Abbreviations

MPSO	3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propane sulfonic acid
BICINE	<i>N,N</i> -bis(2-hydroxyethyl)glycine
Bis-Tris	bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane
BSA	bovine serum albumin
cCMP	cytidine 2',3'-cyclic phosphate (otherwise C>p)
cUMP	uridine 2',3'-cyclic phosphate (otherwise U>p)
DEAE	diethylaminoethyl
EDTA	ethylenediaminetetraacetic acid
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulfonic acid)
IPTG	isopropyl β-D-thiogalactopyranoside
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MOPS	3-(<i>N</i> -morpholino)propanesulfonic acid
MPD	2-methyl-2,4-pentanediol
poly(C)	poly(cytidylic acid)
PAGE	polyacrylamide gel electrophoresis
pdb	Protein Data Bank, which is maintained by the Brookhaven National Laboratory (http://pdb.pdb.bnl.gov/)
ppm	parts per million
RNase A	bovine pancreatic ribonuclease A
sD121A RNase A	semisynthetic RNase A consisting of a noncovalent complex between residues 1 – 118 and residues 111 – 124, with Asp121 replaced by an alanine residue
sD121N RNase A	semisynthetic RNase A consisting of a noncovalent complex between residues 1 – 118 and residues 111 – 124, with Asp121 replaced by an asparagine residue
SDS	sodium dodecyl sulfate
TB	terrific broth
Tris	tris(hydroxymethyl)methane
3'-UMP	uridine 3'-phosphate (otherwise Up)
UpA	uridylyl(3'→5')adenosine.

asparagine or alanine residue. Here, we report on the three-dimensional structures of the variants, as well as their abilities to catalyze transphosphorylation and hydrolysis.

EXPERIMENTAL PROCEDURES

Materials

Escherichia coli strain BL21(DE3) (F^- ompT r_B^- -mB $^-$)(37) was from Novagen (Madison, WI). Buffers (except Tris), cUMP, and IPTG were from Sigma Chemical (St. Louis, MO). Tris was from Fisher Scientific (Pittsburgh, PA). Poly(C) was from Midland Reagent (Midland, TX). UpA was synthesized by J. E. Thompson using methods published previously (38,39). Growth media were from Difco (Detroit, MI) or, for 10-L growths, Marcor Development (Hackensack, NJ). DNA sequences were determined with the Sequenase Version 2.0 kit from U.S. Biochemicals (Cleveland, OH). DEAE Sephadex A-25 anion exchange resin, S-Sepharose cation exchange resin, and the mono-S FPLC cation exchange column were from Pharmacia LKB (Piscataway, NJ). Bacterial terrific broth [TB; (40)] contained (in 1 L) tryptone (12 g), yeast extract (24 g), glycerol (4 mL), KH_2PO_4 (2.3 g), and K_2HPO_4 (12.5 g).

Mutagenesis

Previously, we described the construction of a bovine pancreatic cDNA library, the cloning of the cDNA that codes for RNase A, and the efficient expression of this cDNA in *Escherichia coli* (41). Here, the codon for Asp121 was changed to one for an asparagine or alanine residue by oligonucleotide-mediated site-directed mutagenesis (42) using the oligonucleotides: 5'-ACTACTACACGCTAGCGTTAAAGTGGACT-3' and 5'-ACTACTACACGCTAGCGGCAAAGTGGACT-3', where the underlined bases differ from those in the wild-type cDNA. The GCT mismatch introduced a unique, translationally silent *NheI* site that was used to screen for mutated plasmids. The complete sequence of the cDNA that codes for each enzyme was determined. Plasmids that direct the expression of wild-type, D121N, and D121A RNase A were transformed into *E. coli* BL21(DE3).

Production of RNase A in *E. coli*

TB (0.20 L) containing ampicillin (200 μ g/mL) was inoculated with a culture frozen in aqueous glycerol (30% w/v). After growth overnight at 37 °C, cells were collected by centrifugation and suspended in 0.20 L of fresh TB. This suspension was used to inoculate 10 L of TB containing ampicillin (200 μ 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 0.10 L of cold TE buffer, and lysed by passage through a French pressure cell twice. The lysate was centrifuged at 30000g for 30 min. The resulting pellet was washed with a cold solution (0.30 L) of deoxycholate (1 mg/mL), and centrifuged again. The pellet was suspended in 0.50 L of Tris-acetic acid buffer, pH 8.0, containing urea (9 M), acetic acid (50 mM), sarcosine (40 mM), and EDTA (1 mM). Sonication was used to aid dissolution. To reduce any disulfide bonds, dithiothreitol was added to a final concentration of 50 mM. After 1 h, the solution was passed through DEAE A-25 resin (25 g) that had been equilibrated with the above resuspension solution, and acetic acid (one tenth volume) was added to the effluent. The resulting solution was dialyzed exhaustively against 20 mM acetic acid. The dialysate was centrifuged, the supernatant was diluted to 1 L, and the pH raised to 8.1 by the addition of Tris base. Reduced glutathione and oxidized glutathione were then added to final concentrations of 2.3 mM and 0.8 mM, respectively. After 24 h the resulting solution was loaded on to an S-Sepharose cation exchange column (0.20 L). The column was washed with 1 L of 50 mM HEPES buffer, pH 7.9, and the adsorbed proteins were eluted with a linear gradient (0.50 L + 0.50 L) of NaCl (0 – 0.50 M). Fractions were pooled on the basis of A at 280 nm, and the pooled fractions were diluted with buffer to a protein concentration of

<10 mg/mL. The resulting solution was loaded onto an HR10/10 mono-S cation exchange FPLC column (15 cm × 1.8 cm²) that had been equilibrated with 50 mM HEPES buffer, pH 7.9. The column was washed with 50 mM HEPES buffer, pH 7.9, and the adsorbed proteins were eluted with a linear gradient (50 mL + 50 mL) of NaCl (0 M – 0.50 M) in the same buffer. The proteins eluted between 0.09 M and 0.12 M NaCl. Fractions were again pooled on the basis of *A* at 280 nm, which was monitored continuously. To discourage deamination, the pH of the pooled fractions was lowered to 5.5 by the addition of 20 mM acetic acid. After exhaustive dialysis against water, the proteins were concentrated with an Amicon Centriprep-10® filter, passed through a 0.45-µm filter (Schleicher & Schuell; Keene, NH), and stored at 4 °C.

The purity of the protein preparation was assessed by SDS-PAGE and native PAGE. Based on the symmetry of *A*₂₈₀ peaks during cation exchange chromatography and the appearance of bands after electrophoresis through polyacrylamide gels, wild-type and the D121N variant were ≥99% pure and the D121A variant was ≥95% pure. The concentration of purified enzyme was determined by assuming that *A* = 0.72 at 277.5 nm for a 1.0 mg/mL solution (43). A 10-L growth typically yielded several hundred milligrams of purified enzyme.

Crystallization

Salt-free aqueous solutions of the D121N and D121A variants were lyophilized. Lyophilized D121N RNase A was dissolved in unbuffered water to a concentration of 60 mg/mL.

Crystallization was performed in small siliconized tubes using the batch method. A solution (25 µL) of enzyme was mixed with an equal volume of 95% (v/v) 2-methyl-2,4-pentanediol (MPD) in 0.10 M MES buffer, pH 5.2, to give a final enzyme concentration of 30 mg/mL in 47.5% (v/v) MPD and 0.05 M MES buffer, pH 5.2. The pH of the buffer was determined prior to adding the MPD. Monoclinic crystals of D121N RNase A appeared after several months of incubation at 20 °C. These crystals belong to space group P2₁ with *a* = 30.52 Å, *b* = 38.40 Å, *c* = 53.31 Å, and β = 105.7°.

Lyophilized D121A RNase A was dissolved in unbuffered water to a concentration of 60 mg/mL. Crystallization was performed using the hanging drop method. Drops consisting of enzyme solution (1.5 µL), water (1.5 µL), and reservoir solution (3.0 µL) were suspended from siliconized coverslips over 0.50 mL of 2.5 M sodium acetate buffer, pH 5.3, containing saturating NaCl. Trigonal crystals of the D121A RNase A appeared within one day of incubation at 20 °C, and grew to a size of 0.5 mm × 0.5 mm × 1.0 mm after several days. These crystals belong to space group P3₂21 with *a* = *b* = 64.70 Å, *c* = 65.03 Å, and γ = 120.

X-Ray Diffraction Data Collection

All X-ray data were collected with a Siemens HI-STAR detector mounted on a Rigaku rotating anode operating at 50 kV, 90 mA and a 300 µm focal spot. The X-ray beam was collimated by double focusing mirrors. The crystal to detector distance was 12.0 cm. Data were obtained in 512 × 512 pixel format, processed with the program XDS (44,45) and scaled using the program XSCALIBRE (G. Wesenberg and I. Rayment, unpublished results). The data set for D121N was 88% complete in the resolution range of 30 Å – 1.6 Å with an overall merging *R*-factor of 1.9%. The data set was 91% complete in the resolution range of 30 Å – 1.6 Å with an overall merging *R*-factor of 2.9%.

Structure Refinement

The starting model for the structure of D121N RNase A consisted of residues 2 – 124 of the wild-type enzyme (pdb entry 7rsa), stripped of all solvent molecules and sidechain atoms of residue 121. The model was subjected to ten cycles of least-squares refinement with the program TNT (46), yielding an initial *R*-factor of 23.4%. A difference fourier map (*F*_o – *F*_c)

clearly showed the position of the sidechain atoms of Asn121. Manual adjustments to the model were performed by using the program FRODO (47). After several cycles of manual adjustments and least-squares refinement, the resolution was extended to 1.6 Å and water molecules were added to the model. The peak searching algorithm in TNT was used to place ordered water molecules. Water molecules with at least 1σ of $2F_o - F_c$ density, $3\sigma F_o - F_c$ density and within hydrogen bonding distance of the protein or of other water molecules were retained.

The structure of the D121N variant of RNase A was solved in the monoclinic space group $P2_1$ and contains only water molecules in the active site. Here, the D121N structure will be compared to that of phosphate-free wild-type RNase A, which was also crystallized from organic solvent in the $P2_1$ space group [pdb entry 7rsa; (48)]. The D121N structure will also be compared with the semisynthetic D121N RNase A analog (sD121N), which contains a sulfate ion in the active site and was crystallized from high salt in the trigonal space group $P3_221$. The sD121N structure was solved to a resolution of 2.0 Å [pdb entry 3srn; (30)]. In a least-squares superposition, the mainchain atoms of D121N RNase A have an average RMS deviation of 0.15 Å from those of the wild-type enzyme and 0.75 Å from the sD121N analog.

The starting model for the structure of D121A RNase A consisted of residues 1 – 124 of wild-type RNase A (pdb entry 1rph), stripped of all solvent molecules and sidechain atoms of residue 121. The model was subjected to ten cycles of least-squares refinement with the program TNT (46), yielding an initial *R*-factor of 22.5%. A difference fourier map ($F_o - F_c$) clearly showed the position of the sidechain carbon of Ala121. Manual adjustments to the model were performed by using the program FRODO (47). After several cycles of manual adjustments and least-squares refinement, the resolution was extended to 1.6 Å and water molecules were added to the model. The peak searching algorithm in TNT was used to place ordered water molecules. Water molecules with at least 1σ $2F_o - F_c$ density, $3\sigma F_o - F_c$ density and within hydrogen bonding distance of the protein or of other water molecules were retained.

The structure of the D121A variant of RNase A was solved in the trigonal space group $P3_221$ and contains the first example of an acetate ion in an RNase A active site. Here, the D121A structure will be compared to that of wild-type RNase A that was also crystallized from high salt in the $P3_221$ space group and contains a bound sulfate in the active site [pdb entry 1rph; (49)]. The D121A structure will also be compared with that of the semisynthetic D121A RNase A analog (sD121A), which contains a sulfate ion in the active site and was crystallized from high salt in the $P3_221$ space group. The sD121A structure was solved to a resolution of 2.0 Å [pdb entry 4srn; (30)]. In a least-squares superposition, the mainchain atoms of D121A RNase A have an average RMS deviation of 0.38 Å from those of the wild-type enzyme and 0.53 Å from the sD121A analog.

Enzyme Kinetics

Steady-state kinetic parameters for the cleavage of UpA and poly(C), and the hydrolysis of cUMP were determined by spectrophotometric assays. The catalytic dyad of RNase A most resembles the well-characterized catalytic triad of serine proteases during the hydrolysis reaction (Figure 1). For this reason, the steady-state kinetic parameters for the hydrolysis reaction were evaluated as a function of pH.

Assays were performed at 25 °C in a 0.10 M solution of the appropriate buffer, adjusted in pH with aqueous NaOH or HCl. Enough NaCl was added to each reaction to increase the ionic strength to $I = 0.10$ M, which was calculated based on the concentrations of buffer components and substrate. The buffer used for assays of the transphosphorylation of UpA (0.10 – 1.0 mM) and poly(C) (0.050 – 1.0 mM) was MES (pH 6.0). The buffers used for assays of the hydrolysis of cUMP were formic acid (pH 3.45, 4.00), acetic acid (4.48, 5.02), MES (5.53, 6.03), Bis-Tris (6.00, 7.02), BICINE (6.48, 7.97, 8.46), MOPS (6.94, 7.46), and AMPSO (9.00).

Assays were performed in a 1.0- or 0.2-cm cuvette with a Cary Model 3 spectrophotometer equipped with a Cary temperature controller. Substrate concentrations were determined by ultraviolet absorption using $\epsilon_{260} = 24,600 \text{ M}^{-1}\text{cm}^{-1}$ at pH 7.0 for UpA (50) and $\epsilon_{268} = 6200 \text{ M}^{-1}\text{cm}^{-1}$ at pH 7.8 for poly(C) (51), or by mass for cUMP. The concentrations of substrate in the assays ranged from $K_m/5$ to $5 \times K_m$. BSA (0.5 mg/mL) was added to the reactions containing poly(C). The cleavage of UpA was monitored at 286 nm using $\Delta\epsilon = -755 \text{ M}^{-1}\text{cm}^{-1}$. The cleavage of poly(C) was monitored at 238 nm. The $\Delta\epsilon$ at 250 nm, as calculated from the difference in molar absorptivity of the polymeric substrate and the mononucleotide cyclic phosphate product, has been reported to be $2380 \text{ M}^{-1}\text{cm}^{-1}$ at pH 6.2 (51). The $\Delta\epsilon$ at 238 nm was determined to be $2792 \text{ M}^{-1}\text{cm}^{-1}$ by observing the change in absorption of a partially cleaved substrate at 250 nm and 238 nm. The hydrolysis of cUMP was monitored at either 282 nm or 293 nm. The values for the extinction coefficients determined for the hydrolysis of cUMP are listed in Table 3.

Kinetic Data Analysis

Data processing and nonlinear regression analysis were performed with the program MATHCAD (MathSoft; Cambridge, MA). Initial velocity data were weighted in proportion to the magnitude of each data point. The pH-rate profiles were fitted by using the logarithm of the kinetic constants. Reported errors were generated by the asymptotic variance – covariance matrix method. Because of strong product inhibition and a relatively small change in extinction coefficient, the initial velocities for the hydrolysis of cUMP were difficult to measure reliably. For this reason the steady-state kinetic parameters for this substrate were determined by using the integrated rate equation (52,53) to fit the complete time-course of each hydrolysis reaction. The use of the integrated rate equation provided more accurate values for the steady-state kinetic parameters as well as values for K_p , the dissociation constant for the RNase A•3'-UMP complex. Each progress curve for the hydrolysis reaction was fitted 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 slightly from progress curve to progress curve to allow for small errors in enzyme concentration, with the values of K_m and K_p being held constant. The derivative of the functions so obtained was used to determine better fitting 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

Oligonucleotide-mediated site-directed mutagenesis was used to change Asp121 of the His...Asp catalytic dyad of RNase A to an asparagine or alanine residue. The enzyme variants were produced in *E. coli*, purified, and crystallized. The statistics for X-ray diffraction analysis are listed in Table 1, and the structures of the active sites are shown in Figures 2 and 3.

The final model for D121N contains the complete protein (residues 1 – 124) and 102 water molecules. The *R*-factor for all data in the range 30 to $1.6 \text{ \AA}$ is 0.177. The RMS deviations for target geometries are $0.011 \text{ \AA}$ for bond lengths and 2.1° for bond angles. Average *B*-factors for the mainchain and all atoms are 16.5 and $22.1 \text{ \AA}^2$, respectively. Atomic coordinates for D121N RNase A have been deposited in the pdb with accession code 3rsd.

The final model for D121A contains the complete protein (residues 1 – 124), 100 water molecules, 2 chloride ions, and an acetate ion. The current *R*-factor for all data in the range 30 to $1.6 \text{ \AA}$ is 0.180. The RMS deviations for target geometries are $0.013 \text{ \AA}$ for bond lengths and 2.7° for bond angles. Average *B*-factors for the mainchain and all atoms are 23.8 and 28.4

Å², respectively. Atomic coordinates for D121A RNase A have been deposited in the pdb with accession code 4rsd.

The steady-state kinetic parameters for the turnover at pH 6.0 of UpA, poly(C), and cUMP by wild-type RNase A and the D121N and D121A variants are listed in Table 2. The variations primarily affect the values of k_{cat} . For all three substrates, the k_{cat} for D121A RNase A is approximately 6-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 listed in Table 3. These data are plotted for k_{cat}/K_m , k_{cat} , and K_p in Figures 4 – 6. The solid lines in Figures 4 – 6 are drawn according to the following equations:

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

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

$$K_p = \frac{K_p^{\text{int}}(1 + K_a/[\text{H}^+])(1 + K_b/[\text{H}^+])(1 + [\text{H}^+]/K_p)}{(1 + K_{a,p}/[\text{H}^+])(1 + K_{b,p}/[\text{H}^+])(1 + [\text{H}^+]/K_{p,p})} \quad (3)$$

where $[\text{H}^+]$ is the hydrogen ion concentration; $k_{\text{cat}}/K_m^{\text{int}}$, $k_{\text{cat}}^{\text{int}}$, $k_{\text{cat}}^{\text{int2}}$, and K_p^{int} are pH-independent 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 3'-UMP; and those constants with an “s” or “p” subscript are for the enzyme•substrate or enzyme•product complex (52,53). In eq 1, the terms associated with K_c and K_d were not included in the fit when there was no indication of their presence. As no indication for $pK_{p,p}$ was present, it was set at 2.0 (54). The value of pK_p was set at 5.8 (55). The free enzyme values of K_p were obtained from the k_{cat}/K_m profiles. The pK_a values and intrinsic parameters calculated from eq 1 – 3 are listed in Table 4.

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

As shown in Figure 5, wild-type RNase A, D121N RNase A, and D121A RNase A have similar k_{cat} profiles at pH < 7, though that of the D121N enzyme is somewhat shallower than that of the others. At pH > 7, the k_{cat} profile of the wild-type enzyme reaches a plateau, but those of the variants decrease. The k_{cat} profiles for wild-type RNase A and D121N RNase A have slope = 1 at pH < 4.5; all three enzymes have slope < 1 between pH 4.5 and 7.0.

As shown in Figure 6, the K_p profile 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 enzymes.

DISCUSSION

Three-Dimensional Structures

RNase A was the third enzyme (after lysozyme and carboxypeptidase) whose structure was solved by X-ray diffraction analysis (56). Despite this early precedent (57), the crystalline structures reported here are the first for active-site variants. These structures show that replacing residue Asp121 of the His···Asp catalytic dyad with an asparagine or alanine residue does not affect the structure of RNase A beyond residue 121. Interpretations of function based on the identity of the active-site residues alone are thus justified.

The structure of RNase A is comprised of a twisted four-stranded, antiparallel β -sheet consisting of two long central β -strands, $\beta 2$ and $\beta 3$ (residues 71 – 92 and 94 – 110, respectively), flanked by two shorter β -strands, $\beta 1$ and $\beta 4$ (residues 41 – 48 and 118 – 123, respectively). Three helices are found in RNase A: $\alpha 1$ at the N-terminus (residues 3 – 13), and $\alpha 2$ and $\alpha 3$ nearly perpendicular to and at either end of the β -sheet (residues 24 – 34 and 50 – 60, respectively). Nucleotide binding occurs in a deep cleft created by $\alpha 1$ and $\beta 3$ (58). The bases of adjacent RNA nucleotides bind in three enzymic subsites referred to as B1, B2, and B3 (59). Catalysis occurs in the P1 subsite and results in the cleavage of the P–O^{5'} bond specifically on the 3'-side of a pyrimidine nucleotide bound in the B1 subsite. Residues from the B1 subsite and P1 catalytic site compose the walls and floor of the groove. Gln11, Lys41, and Asn44 form one wall, and His119, Asp121, and Ala122 form the other. His12, Asn44, Thr45, and Phe120 form the floor of the B1 subsite. This discussion will focus on structural deviations observed in residues constituting the active site (Figures 2 and 3).

Gln11

The sidechain N_{ε2} of Gln11 can donate a hydrogen bond to a phosphoryl oxygen in the active site. Site-directed mutagenesis studies in which Gln11 is replaced by an alanine, asparagine, and histidine residue have shown that the role of Gln11 is to prevent the nonproductive binding of substrate (41). In the structure of the RNase A•uridine 2',3'-cyclic vanadate complex, N_{ε2} of Gln11 forms a hydrogen bond with a nonbridging vanadyl oxygen, perhaps mimicking its interaction with the transition state (58,60). The sidechain N_{ε2} of Gln11 forms a hydrogen bond with a bridging phosphoryl oxygen in an RNase A•d(CpA) complex, and with a water molecule in an RNase A•3'-CMP complex (49).

In the wild-type monoclinic structure, N_{ε2} of Gln11 forms a 3.0 Å hydrogen bond with a conserved water molecule in the active site, but O_{ε1} forms no hydrogen bonds to surrounding water molecules (48). In the structure of D121N RNase A, the sidechain of Gln11 adopts a slightly different conformation. The sidechain N_{ε2} of Gln11 forms a longer (3.4 Å) hydrogen bond with the conserved active-site water molecule, and O_{ε1} forms a 2.9 Å hydrogen bond with a new water molecule. In the sD121N analog, N_{ε2} of Gln11 adopts the same conformation as in wild-type RNase A, forming a 2.8 Å hydrogen bond with the conserved active-site water molecule.

In the wild-type trigonal structure, N_{ε2} of Gln11 forms a 2.6 Å hydrogen bond with the conserved active-site water and a 3.0 Å hydrogen bond with a sulfate oxygen bound in the active site (49). The conserved active-site water is displaced by C₁ of an acetate ion in the D121A variant. The result is a small shift of N_{ε2} away from the acetate (due to a 30° rotation around χ_3), with O_{ε1} forming a hydrogen bond with a new water molecule. In the sD121A structure, N_{ε2} of Gln11 forms a 2.8 Å hydrogen bond with the conserved active-site water molecule. But unlike in the wild-type enzyme, N_{ε2} in the sD121A analog does not interact with the sulfate bound in the active site.

His12

In the classical mechanism for RNase A catalysis, N_{ε2} of His12 acts as a base to abstract a proton from the 2'-hydroxyl group of RNA and thereby facilitate its attack on the phosphorous atom (33,61). In all structures of RNase A in the pdb, His12 is found in the same position regardless of the crystallization conditions, solvent molecules, or ligand in the active site [(62); L. W. Schultz and R. T. Raines, unpublished results]. His12 forms a hydrogen bond between N_{δ1} of its sidechain and the mainchain carbonyl of Thr45, and between N_{ε2} and a water molecule or a ligand in the active site. The position of His12 in the D121N or D121A variants has not changed, overlapping quite well with His12 from the sD121N and sD121A structures. In the D121N structure, N_{ε2} of His12 forms a 2.7 Å hydrogen bond with a water molecule in the active site. A similar 2.7 Å hydrogen bond is also found in the wild-type monoclinic structure. In the structure of the sD121N analog, N_{ε2} of His12 forms a 2.5 Å bond with a sulfate oxygen in the active site. In the D121A structure, N_{ε2} of His12 forms a 2.7 Å hydrogen bond with an acetate oxygen in the active site. This acetate oxygen is located in the same position as is the sulfate oxygen that forms a 2.8 Å hydrogen bond with N_{ε2} in the wild-type trigonal structure. As in the sD121N analog, His12 of the sD121A analog forms a 2.5 Å hydrogen bond with a sulfate oxygen in the active site.

Lys41

The sidechain of Lys41 has been implicated in transition state stabilization, contributing 10⁵-fold to catalysis (63). In addition, N_ζ of Lys41 has been shown to form a hydrogen bond with the 2'-oxygen of 3'-CMP and to a bridging vanadyl oxygen of uridine 2',3'-cyclic vanadate (58,49). In the D121N and D121A variants, the sidechain of Lys41 has clearly defined density and exists in a fully extended conformation. In the wild-type monoclinic and trigonal structures, N_ζ of Lys41 forms a hydrogen bond (2.6 Å and 2.7 Å, respectively) with O_{δ1} of Asn44. The structures of the D121N and D121A variants have the same hydrogen bond (2.8 Å and 2.9 Å, respectively) between N_ζ of Lys41 and O_{δ1} of Asn44, but N_ζ in the D121A enzyme makes an additional 2.5 Å hydrogen bond with a new water molecule in the active site. In the sD121N and sD121A analogs, N_ζ of Lys41 also forms a hydrogen bond (2.9 Å and 3.2 Å, respectively) to O_{δ1} of Asn44. As in the D121A variant, however, N_ζ of Lys41 in the sD121N analog forms an additional 2.7 Å hydrogen bond with a water molecule in the active site. In the sD121A analog, N_ζ also forms a 2.8 Å hydrogen bond with O_{ε1} of Gln11.

Residues 65 – 72

Residues 65 – 72 form a loop between α3 and β2. This loop resides near the active site and contains Lys66, which is implicated in the binding of a substrate phosphoryl group (59). Lys66 makes two interactions with Asp121 in the wild-type monoclinic RNase A structure: a 2.8 Å hydrogen bond from the mainchain N of Lys66 to O_{δ2} of Asp121 and a 3.0 Å hydrogen bond from N_ζ of Lys66 to the mainchain O of Asp121. In the wild-type trigonal structure, the mainchain N of Lys66 also forms a 3.3 Å hydrogen bond to O_{δ2} of Asp121, but N_ζ of Lys66 forms no interactions with the protein.

The hydrogen bond between the mainchain N of Lys66 and O_{δ2} of Asp121 restricts conformational entropy by linking two parts of the native enzyme that are proximal in space but distal in sequence. ¹H-NMR data suggest that this hydrogen bond is especially strong (64). In X-ray diffraction analyses, the length of this hydrogen bond appears to rely on the crystallization conditions, being 2.8 – 2.9 Å in salt-free crystals and 2.9 – 3.3 Å in crystals containing salt.

Movement of the loop containing residues 65 – 72 away from the active site has been proposed to be a major contributor to the reduced activity of the sD121N and sD121A analogs (30). The mainchain atoms of residues 65 – 72 in the trigonal sD121N structure have an average shift of

0.76 Å away from the wild-type mainchain. In contrast, the same atoms in the monoclinic D121N structure have an average shift of 0.45 Å away from the wild-type mainchain, suggesting that the crystallization conditions and not the amino acid substitution are responsible for the larger shift.

Other significant differences exist between the D121N and sD121N structures (Figure 3A). The hydrogen bond between the mainchain N of Lys66 and O_{δ1} of Asn121 is 2.9 Å in the D121N variant, but 3.2 Å in the sD121N analog. The sidechain N_ε of Lys66 forms a 3.3 Å hydrogen bond with the mainchain O of Asn121 in the D121N variant, but a 3.5 Å hydrogen bond with O_{δ1} of Asn121 in the sD121N analog. Semisynthetic F120L and F120Y analogs of RNase A in the trigonal form also form hydrogen bonds between N_ε of Lys66 and O_{δ1} of Asp 121, but neither forms a hydrogen bond with the mainchain O of Asp121 (65). Another difference is more dramatic, as it involves the flipping of the Lys66 – Asn67 peptide bond. The ϕ, ψ angles for Lys66 and Asn67 for the wild-type RNase A ($-56^\circ, -35^\circ$ and $-85^\circ, 1^\circ$) and the D121N variant ($-55^\circ, -39^\circ$ and $-82^\circ, -5^\circ$) are similar, but those for the sD121N analog ($-84^\circ, 83^\circ$ and $166^\circ, -26^\circ$) differ. In the sD121N structure, a water molecule occupies the position of the mainchain O in the D121N structure. This water molecule in the sD121N structure is only 2.1 Å (which is an unrealistic distance) from the mainchain N of Asn67. We therefore suspect that the discrepancy between the location of residues 65 – 72 in the D121N and sD121N structures is largely due to an error in electron density map interpretation.

Residues 65 – 72 in D121A RNase A are almost identical in structure to residues 65 – 72 in the sD121A analog. The loss of the hydrogen bond between the sidechain of residue 121 and the mainchain N of Lys66 has caused the mainchain atoms in residues 65 – 72 in both structures to move away from the wild-type mainchain by an average 0.7 Å. Moreover, the Lys66 sidechain forms no hydrogen bonds to the protein in either structure. The absence of this hydrogen bond is, however, not uncommon [pdb entries 1lsq and 1rnc; (66,67)]. Although the Asp121 sidechain to Lys66 mainchain hydrogen bond appears to be important in stabilizing the structure of residues 65 – 72, it is unlikely that hydrogen bonds to the sidechain of Lys66 play a significant role in that stabilization.

His119

In the classical mechanism of RNase A catalysis (Figure 1), His119 acts as a general acid by donating a proton to the 5'-oxygen to facilitate its displacement (33,61). The sidechain of His119 has been shown to occupy two distinct positions, denoted as A and B, which are related by a rotation around χ_1 to dihedral angles of approximately 159° and -60° , respectively, and a concurrent rotation around χ_2 of 180° (68,69). The active-site histidine residue in carbonic anhydrase II undergoes a similar rotation (70). There has been considerable debate as to which position of His119 in RNase A is catalytically relevant because the imidazole nitrogens have access to the active site in both orientations (30). It has also been argued that His119 is in position A during catalysis of transphosphorylation and in position B during catalysis of hydrolysis (69). In position A, N_{ε2} of His119 forms a hydrogen bond with O_{δ1} of Asp121, which directs N_{δ1} of His119 toward the active site where it can form a hydrogen bond with a ligand (49). In the absence of a ligand, N_{δ1} of His119 forms no apparent hydrogen bonds to water molecules (48). In the RNase A•3'-CMP complex, the sidechain of His119 is in position B, bringing N_{ε2} within 3 Å of a nonbridging phosphoryl oxygen of 3'-CMP. In position B, N_{δ1} of His119 is distal from the active site. In the crystalline RNase A•d(CpA) complex, the B2 subsite is occupied by adenine and the His119 sidechain is in position A (49). If the His119 sidechain were in position B, the imidazole ring would be in direct contact with the purine base and would create a steric clash (L.W. Schultz and R.T. Raines, unpublished results). It is therefore unlikely that His119 catalyzes RNA transphosphorylation reactions from position B.

The sidechain of His119 occupies position A in the D121N structure and overlaps with His119 in the wild-type structure almost exactly, with $\chi_1 = 160^\circ$. The sidechain $N_{\delta 2}$ of His119 forms a 2.6 Å hydrogen bond with $N_{\delta 2}$ of Asn121, and $N_{\delta 1}$ forms no hydrogen bonds. Similarly, the sidechain of His119 occupies position A in the D121A structure but has shifted toward the active site by a 15° rotation about $\chi_1 = 175^\circ$. In the absence of a hydrogen-bonding sidechain at residue 121, $N_{\epsilon 2}$ of His119 forms no hydrogen bonds, and $N_{\delta 1}$ of His119 forms a 2.5 Å hydrogen bond with the acetate ion in the active site. In the sD121A ($\chi_1 = -50^\circ$) and sD121N ($\chi_1 = -49^\circ$) analogs, the sidechain of His119 occupies position B. Others have concluded that in the absence of a hydrogen bond with the sidechain of Asp121, His119 would prefer position B over position A (30). We too expected a priori that replacement of Asp121 would shift the $B \rightleftharpoons A$ equilibrium in favor of position B because of the loss of the favorable His...Asp interaction. Yet in the structures of crystalline D121N RNase A and D121A RNase A, His119 is in position A (Figures 2 and 3). It appears that the position of His119 is more dependent on crystallization conditions than on the identity of residue 121. Similarly, the position of His119 has been shown to depend on the pH (65) and ionic strength (62) of the crystallization solution. Finally, unlike in serine hydrolases (71), $C_{\epsilon 1}$ of His119 is not proximal to a mainchain O in either position A or position B. (Likewise, $C_{\epsilon 1}$ of His12 is not near a mainchain O.)

Asn121

At a resolution of 1.6 Å, it is impossible to distinguish $N_{\delta 2}$ and $O_{\delta 1}$ in the sidechain of Asn121. It is, however, possible to distinguish these atoms based on their hydrogen bonding schemes. In our model, $O_{\delta 1}$ of Asn121 occupies a position similar to that of $O_{\delta 2}$ in wild-type RNase A, forming a 2.9 Å hydrogen bond with the mainchain N of Lys66. This orientation is sensible because it is unlikely that $N_{\delta 2}$ of Asn121 could be in close proximity to a mainchain nitrogen. This orientation places $N_{\delta 2}$ of Asn121 near $N_{\epsilon 2}$ of His119. At pH 5.3 (which is the pH of the crystallization buffer), $N_{\epsilon 2}$ of His119 should be protonated, thereby rendering its interaction with the Asn121 sidechain unfavorable. But, the crystals of D121N RNase A were grown in MPD, a less polar solvent than water. Accordingly, the pK_a of His119 in the crystal is likely to be less than in water. We therefore propose that His119 is in the neutral imidazole state in which $N_{\delta 1}$ is protonated, and that $N_{\epsilon 2}$ forms a 2.6 Å hydrogen bond with the sidechain of Asn121.

A water molecule forms a bridging hydrogen bond between $N_{\delta 2}$ of Asn121 and the mainchain N of Asn67. The $N_{\delta 2}$ – water hydrogen bond distance is 3.1 Å with an $N_{\delta 2}$ – H – O angle of 120° . This water molecule, which is conserved in several RNase A structures, could stabilize the conformation of residues 65 – 72 by linking them to residue 121. In the sD121N analog, the water molecule has moved significantly, forming a long hydrogen bond with the mainchain N of Asn67 and perhaps resulting in a shift of the residues 65 – 72 away from the active site. Still, the sidechains of Asn121 in the D121N and sD121N structures overlap almost exactly (Figure 3A).

Ala121

The mainchain near residue 121 in the D121A variant remains in the same conformation as in the trigonal wild-type structure. The sidechain C_β of Ala121 overlaps directly with C_β of Asp121 in the wild-type RNase structure. In the D121A variant, a water molecule takes the place of $O_{\delta 2}$ of Asp121 and forms a 3.2 Å hydrogen bond with the mainchain N of Lys66. This water molecule is absent from the sD121A structure. In addition, the conserved water molecule that forms a bridging hydrogen bond between $O_{\delta 1}$ of Asp121 and the mainchain N of Asn67 in the wild-type enzyme is missing in the D121A and sD121A structures.

Catalysis by D121N RNase A and D121A RNase A

The effect of replacing Asp121 on $k_{\text{cat}}/K_{\text{m}}$ and k_{cat} is small, but significant (Tables 2 – 4). Substitution with an asparagine residue decreases the rate constants for transphosphorylation by 10^1 -fold and those for hydrolysis by $10^{0.5}$ -fold (Table 2). Substitution with an alanine residue decreases the rate constants for transphosphorylation by 10^2 -fold and those for hydrolysis by 10^1 -fold (Tables 2 – 4). The differential effects on transphosphorylation and hydrolysis are still greater than these values suggest because a chemical step does not limit the rate of cleavage of UpA or poly(C) (72). We conclude that the contribution of Asp121 to transphosphorylation is greater than that to hydrolysis.

The small contribution of Asp121 to catalysis belies the importance of the hydrogen bond between His119 and Asp121. This contribution is similar to that observed in an analogous study on the structure and function of phosphatidylinositol-specific phospholipase C (73). Yet, a similar His $\cdots$ Asp dyad has been proposed to form a low-barrier hydrogen bond of extraordinary strength during catalysis by the protease chymotrypsin (74,75), though this interpretation is controversial (76). One criterion for such a bond is an ^1H chemical shift of 17 – 20 ppm (77). The ^1H chemical shift of $\text{N}_{\epsilon 2}\text{H}$ of His119 appears at a much higher field, <12 ppm (J. L. Markley, personal commun.). By this criterion, the His $\cdots$ Asp dyad of RNase A does not have a low-barrier hydrogen bond. In theory, the His $\cdots$ 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 $\text{p}K_{\text{a}}$ values (79). The $\text{p}K_{\text{a}}$ values of acetamide [15.1; (80)] and imidazole [14.2; (81)], which are reasonable models for the sidechains of His119 and Asn121, respectively, are indeed similar. The existence of a low-barrier hydrogen bond would, however, require that the sidechain of His119 be in its neutral imidazole form. pH–rate profiles for catalysis by D121N RNase A (Figures 4–6) and ^1H NMR titrations of His119 in the D121N variant (54) indicate that His119 titrates between its imidazole and imidazolium forms with a $\text{p}K_{\text{a}}$ value similar to that of His119 in wild-type RNase A. These data deny the existence of a low-barrier hydrogen bond in the His $\cdots$ Asn dyad.

The kinetics of catalysis by D121N RNase A and D121A RNase A illuminate mechanistic aspects of the $\text{B} \rightleftharpoons \text{A}$ equilibrium of His119. A Coulombic interaction, like that between a cationic histidine sidechain and an anionic aspartate sidechain, has a long range. In contrast, a hydrogen bond, like that between a histidine sidechain and an aspartate or asparagine sidechain, has a short range. Dispersion interaction between a histidine sidechain and an alanine sidechain are insignificant at distance beyond van der Waals radii. We find that RNase A variants with an aspartate, asparagine, or alanine residue in position 121 have different abilities to catalyze the hydrolysis reaction (Tables 3 and 4). We therefore conclude that His119 is likely to be proximal to residue 121 during catalysis of hydrolysis, and that hydrolysis can occur with His119 in position A.

pH-Dependence of Catalysis

The overall shape of the pH–rate profiles for catalysis by RNase A can be interpreted by using a model in which the only important protonation states are those of the two active-site histidine residues, which act in concert during catalysis [Figure 1; (82)]. Accordingly, log–log plots of the pH–rate profiles for $k_{\text{cat}}/K_{\text{m}}$ and k_{cat} are expected to be bell-shaped with slopes approaching unity. The $k_{\text{cat}}/K_{\text{m}}$ profiles that have been reported are indeed bell-shaped, but the shape of the k_{cat} profiles varies with the substrate (83–86). In general, workers who have used cCMP as the substrate have found that the slope = 1 for the acidic leg of the k_{cat} profiles; but those who have used cUMP have found that the slope < 1. The large values of K_{m} and strong product inhibition observed for the hydrolysis step at high pH make the basic leg difficult to characterize. These factors are likely to be the cause of the discrepant values reported for k_{cat} at high pH (83).

Our data on the pH-dependence of $k_{\text{cat}}/K_{\text{m}}$ for the hydrolysis of cUMP agree with those of previous studies. [For a review, see: (87).] Our data on the pH-dependence of k_{cat} for the hydrolysis of cUMP are most similar to those of workers who, like us, accounted for product inhibition by using the integrated rate equation (86). This approach yields a slope near one at values of pH < 4.5, as well as higher values of k_{cat} and K_{m} at values of pH > 7 than are reported by others (84,85).

Values of $\text{p}K_{\text{a}}$ for the two histidine residues of wild-type RNase A can be derived from the $k_{\text{cat}}/K_{\text{m}}$ profile (Table 4). The values agree well with results from NMR spectroscopy (88-91, 54). Differences for any given $\text{p}K_{\text{a}}$ value are within error, especially when recalling that when two $\text{p}K_{\text{a}}$'s have similar values, the average value is much better determined from pH-rate profiles than are the individual values (92). The $\text{p}K_{\text{a}}$ values of the D121N and D121A enzymes that are derived from pH-rate profiles (Table 4) do not deviate significantly from those of wild-type RNase A, at least at $I = 0.10 \text{ M}$.

Role of Asp121 in Catalysis

pH-rate profiles for catalysis by D121N RNase A are similar to those for catalysis by the wild-type enzyme (Figures 4 – 6). This similarity suggests that the same titratable groups are participating in catalysis and that these groups have similar $\text{p}K_{\text{a}}$ values. Yet in the structure of D121N RNase A, $\text{N}_{\delta 2}$ rather than $\text{O}_{\delta 1}$ of Asn121 is oriented toward His119. This orientation appears to stabilize a neutral form of the imidazolyl sidechain in which $\text{N}_{\epsilon 2}$ is unprotonated and $\text{N}_{\delta 1}$ is protonated. This tautomer of His119 is likely to be ineffective, either as an acid during the transphosphorylation reaction or as a base during the hydrolysis reaction. The major role of Asp121 thus appears to be to orient the proper tautomer of His119 for catalysis. This role is similar to that assigned to the aspartate residue in the catalytic triad of trypsin (26). In the D121A enzyme, Ala121 cannot interact with the imidazolyl group of His119. Yet, D121N RNase A is a better catalyst than is D121A RNase A (Tables 2 – 4). Thus, the His119...Asn121 interaction observed in the crystalline D121N enzyme (Figures 2 and 3) is unlikely to exist in solution. Rather, Asn121 is likely to move such that His119 can effect catalysis.

Conclusions

Replacing Asp121, a residue that is conserved in pancreatic ribonucleases, with an asparagine or alanine residue has no effect on the overall three-dimensional structure of RNase A. Replacing Asp121 decreases the rate of transphosphorylation by $\leq 10^2$ -fold and the rate of hydrolysis by $\leq 10^1$ -fold. Thus, Asp121 of RNase A makes a significant but not a substantial contribution to catalysis. The likely role of Asp121 is simply to position the proper tautomer of His119, which Asn121 in the crystalline D121N enzyme fails to do. We speculate that the role of Asp121 in catalysis alone is unlikely to warrant its complete conservation in pancreatic ribonucleases (54).

Acknowledgments

We thank B. R. Kelemen, P. A. Leland, and B. M. Templer for comments on the manuscript, and Prof. J. L. Markley for advice. We are grateful to Prof. I. Rayment, Prof. H. M. Holden, and the members of their research groups for many helpful conversations and for the use of their X-ray data collection and computational facilities.

REFERENCES

1. Neurath, H. *Proteolytic Enzymes: A Practical Approach*. Beynon, RJ.; Bond, JS., editors. IRL Press; New York: 1989. p. 1-13.
2. Blow DM, Birktoft JJ, Hartley BS. *Nature* 1969;221:337–340. [PubMed: 5764436]
3. Blow DM. *Trends Biochem. Sci* 1997;22:405–408. [PubMed: 9357317]
4. Neurath H. *Science* 1984;224:350–357. [PubMed: 6369538]

5. Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JH, Christiansen L, Huge-Jensen B, Norskov L, L. T, Menge U. *Nature* 1990;343:767–770. [PubMed: 2304552]
6. Winkler FK, D’Arcy A, Hunziker W. *Nature* 1990;343:771–774. [PubMed: 2106079]
7. Martinez C, De Geus P, Lauwereys M, Matthyssens G, Cambillau C. *Nature* 1992;356:615–618. [PubMed: 1560844]
8. Choi H-K, Tong L, Minor W, Dumas P, Boege U, Rossmann MG, Wengler G. *Nature* 1991;354:37–43. [PubMed: 1944569]
9. Faustinella F, Chang A, Van Biervliet JP, Rosseneu M, Vinaimont N, Smith LC, Chen S-H, Chan L. *J. Biol. Chem* 1991;266:14418–14424. [PubMed: 1907278]
10. Liao D-I, Breddam K, Sweet RM, Bullock T, Remington SJ. *Biochemistry* 1992;31:9796–9812. [PubMed: 1390755]
11. Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I. *Science* 1991;253:872–879. [PubMed: 1678899]
12. Li Y, Tsai M-D. *J. Am. Chem. Soc* 1993;115:8523–8526.
13. Verschuere KHG, Seljée F, Rozeboom HJ, Kalk KH, Dijkstra BW. *Nature* 1993;363:693–698. [PubMed: 8515812]
14. Pathak D, Ollis D. *J. Mol. Biol* 1990;214:497–525. [PubMed: 2380986]
15. Christianson DW, Alexander RS. *J. Am. Chem. Soc* 1989;111:6412–6419.
16. Beintema JJ. *Life Chem. Rep* 1987;4:333–389.
17. Beintema JJ, Schüller C, Irie M, Carsana A. *Prog. Biophys. Molec. Biol* 1988;51:165–192. [PubMed: 3074337]
18. Gandour RD. *Bioorg. Chem* 1981;10:169–176.
19. Zimmerman SC, Cramer KD. *J. Am. Chem. Soc* 1988;110:5906–5908.
20. Cramer KD, Zimmerman SC. *J. Am. Chem. Soc* 1990;112:3680–3682.
21. Schowen, RL. *Mechanistic Principles of Enzyme Activity*. Liebman, JF.; Greenberg, A., editors. VCH Publishers; New York: 1988. p. 119-168.
22. Umeyama H, Nakagawa S, Fujii T. *Chem. Pharm. Bull* 1979;27:974–980. [PubMed: 476866]
23. Craik CS, Largman C, Fletcher T, Rocznik S, Barr PJ, Fletterick R, Rutter WJ. *Science* 1985;228:291–297. [PubMed: 3838593]
24. Corey DR, Craik CS. *J. Am. Chem. Soc* 1992;114:1784–1790.
25. Carter P, Wells JA. *Nature* 1988;332:564–568. [PubMed: 3282170]
26. Sprang S, Standing T, Fletterick RJ, Stroud RM, Finer-Moore JF, Xuong NH, Hamlin R, Rutter WJ, Craik CS. *Science* 1987;237:905–909. [PubMed: 3112942]
27. Raines RT. *Chem. Rev* 1998;98 In Press.
28. Stern MS, Doscher MS. *FEBS Lett* 1984;171:253–256. [PubMed: 6427012]
29. Cederholm MT, Stuckey JA, Doscher MS, Lee L. *Proc. Natl. Acad. Sci. U.S.A* 1991;88:8116–8120. [PubMed: 1896458]
30. de Mel VSJ, Martin PD, Doscher MS, Edwards BFP. *J. Biol. Chem* 1992;267:247–256. [PubMed: 1730593]
31. Trautwein K, Holliger P, Stackhouse J, Benner SA. *FEBS Lett* 1991;281:275–277. [PubMed: 1901803]
32. Harper JW, Vallee BL. *Proc. Natl. Acad. Sci. U.S.A* 1988;88:7139–7143. [PubMed: 2459697]
33. Findlay D, Herries DG, Mathias AP, Rabin BR, Ross CA. *Nature* 1961;190:781–784. [PubMed: 13699542]
34. Cuchillo CM, Parés X, Guasch A, Barman T, Travers F, Nogués MV. *FEBS Lett* 1993;333:207–210. [PubMed: 7693511]
35. Thompson JE, Venegas FD, Raines RT. *Biochemistry* 1994;33:7408–7414. [PubMed: 8003506]
36. Doolittle RF. *Protein Sci* 1992;1:191–200. [PubMed: 1339026]
37. Studier FW, Moffatt BA. *J. Mol. Biol* 1986;189:113–130. [PubMed: 3537305]

38. Ogilvie KK, Beaucage SL, Schiffman AL, Theriault NY, Sadana KL. *Can. J. Chem* 1978;56:2768–2780.
39. Beaucage SL, Caruthers MH. *Tetrahedron Lett* 1981;22:1859–1862.
40. Tartof KD, Hobbs CA. *Bethesda Res. Lab. Focus* 1987;9:12.
41. delCardayré SB, Ribó M, Yokel EM, Quirk DJ, Rutter WJ, Raines RT. *Protein Eng* 1995;8:261–273. [PubMed: 7479688]
42. Kunkel TA, Roberts JD, Zakour RA. *Methods Enzymol* 1987;154:367–382. [PubMed: 3323813]
43. Sela M, Anfinsen CB, Harrington WF. *Biochim. Biophys. Acta* 1957;26:502–512. [PubMed: 13499407]
44. Kabsch W. *J. Appl Crystallogr* 1988;21:67–71.
45. Kabsch W. *J. Appl. Crystallogr* 1988;21:916–924.
46. Tronrud DE, Ten-Eyck LF, Matthews BW. *Acta Crystallogr., Sect. A* 1987;43:489–501.
47. Jones TA. *Methods Enzymol* 1985;115:157–171. [PubMed: 3841179]
48. Wlodawer A, Anders LA, Sjölin L, Gilliland GL. *Biochemistry* 1988;27:2705–2717. [PubMed: 3401445]
49. Zegers I, Maes D, Thi MH, Poortmans F, Palmer RA, Wyns L. *Protein Sci* 1994;3:2322–2339. [PubMed: 7756988]
50. Warshaw MM, Tinoco I. *J. Mol. Biol* 1966;20:29–38. [PubMed: 5970660]
51. Yakovlev GI, Moiseyev GP, Bezborodova SI, Both V, Sevcik J. *Eur. J. Biochem* 1992;204:187–190. [PubMed: 1310940]
52. Segel, IH. *Enzyme Kinetics*. John Wiley & Sons; New York, NY: 1975.
53. Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*. Portland Press; London: 1995.
54. Quirk, DJ. Ph.D. Thesis. University of Wisconsin–Madison; 1996.
55. Tanokura M. *J. Biochem* 1983;94:1621–1630. [PubMed: 6654875]
56. Kartha G, Bello J, Harker D. *Nature* 1967;213:862–865. [PubMed: 6043657]
57. Gilliland, GL. *Ribonucleases: Structures and Functions*. D'Alessio, G.; Riordan, JF., editors. Academic Press; New York: 1997. p. 306–341.
58. Wlodawer A, Miller M, Sjölin L. *Proc. Natl. Acad. Sci. U.S.A* 1983;80:3628–3631. [PubMed: 6574501]
59. Parés X, Nogués MV, de Llorens R, Cuchillo CM. *Essays Biochem* 1991;26:89–103. [PubMed: 1778187]
60. Ladner JE, Wladkowski BD, Svensson LA, Sjölin L, Gilliland GL. *Acta Crystallogr., Sect. D* 1997;53:290–301. [PubMed: 15299932]
61. Thompson JE, Raines RT. *J. Am. Chem. Soc* 1994;116:5467–5468.
62. Fedorov AA, Joseph-McCarthy D, Fedorov E, Sirakova D, Graf I, Almo SC. *Biochemistry* 1996;35:15962–15979. [PubMed: 8973167]
63. Messmore JM, Fuchs DN, Raines RT. *J. Am. Chem. Soc* 1995;117:8057–8060.
64. Baker WR, Kintanar A. *Arch. Biochem. Biophys* 1996;327:189–199. [PubMed: 8615690]
65. de Mel VSJ, Doscher MS, Martin PD, Edwards BFP. *FEBS Leters* 1994;349:155–160.
66. Capasso S, Giordano F, Mattia CA, Mazzarella L, Zagari A. *Biopolymers* 1983;22:327–332. [PubMed: 6673761]
67. Aguilar CF, Thomas PJ, Mills A, Moss DS, Palmer RA. *J. Mol. Biol* 1992;224:265–267. [PubMed: 1548704]
68. Borkakoti N, Moss DS, Palmer RA. *Acta Crystallogr. B* 1982;38:2210–2217.
69. Borkakoti N. *Eur. J. Biochem* 1983;132:89–94. [PubMed: 6404632]
70. Nair SK, Christianson DW. *J. Am. Chem. Soc* 1991;113:9455–9458.
71. Derewenda ZS, Derewenda U, Kobos PM. *J. Mol. Biol* 1994;241:83–93. [PubMed: 8051710]
72. Thompson JE, Kutateladze TG, Schuster MC, Venegas FD, Messmore JM, Raines RT. *Bioorg. Chem* 1995;23:471–481.
73. Gässler CS, Ryan M, Liu T, Griffith OH, Heinz DW. *Biochemistry* 1997;36:12802–12813. [PubMed: 9335537]

74. Frey PA, Whitt SA, Tobin JB. Science 1994;264:1927–1930. [PubMed: 7661899]
75. Cassidy CS, Lin J, Frey PA. Biochemistry 1997;36:4576–4584. [PubMed: 9109667]
76. Ash EL, Sudmeier JL, De Fabo EC, Bachovchin WW. Science 1997;278:1128–1132. [PubMed: 9353195]
77. Cleland WW, Kreevoy MM. Science 1994;264:1887–1890. [PubMed: 8009219]
78. Patel DJ, Woodward CK, Bovey FA. Proc. Natl. Acad. Sci. U.S.A 1972;69:599–602. [PubMed: 4501576]
79. Cleland WW. Biochemistry 1992;31:317–319. [PubMed: 1731889]
80. Bordwell FG. Acc. Chem. Res 1988;21:456–463.
81. Walba H, Isensee RW. J. Am. Chem. Soc 1955;77:5488–5492.
82. Sowa GA, Hengge AC, Cleland WW. J. Am. Chem. Soc 1997;119:2319–2320.
83. Herries DG, Mathias AP, Rabin BR. Biochem. J 1962;85:127–134. [PubMed: 13954073]
84. delRosario EJ, Hammes GG. Biochemistry 1969;8:1884–1889. [PubMed: 5814934]
85. Machuga E, Klapper MH. Biochim. Biophys. Acta 1977;481:526–41. [PubMed: 15611]
86. Eftink MR, Biltonen RL. Biochemistry 1983;22:5123–5134. [PubMed: 6317013]
87. Richards FM, Wyckoff HW. The Enzymes IV 1971:647–806.
88. Meadows DH, Roberts GCK, Jardetzky O. J. Mol. Biol 1969;45:491–511. [PubMed: 5394188]
89. Roberts GCK, Dennis EA, Meadows DH, Cohen JS, Jardetzky O. Proc. Natl. Acad. Sci. U.S.A 1969;62:1151–1158. [PubMed: 5256413]
90. Markley JL, Finkenstadt WR. Biochemistry 1975;14:3562–3566. [PubMed: 240383]
91. Patel DJ, Canuel LL, Bovey FA. Biopolymers 1975;14:987–997. [PubMed: 1156653]
92. Brocklehurst K. Protein Eng 1994;7:291–299. [PubMed: 8177877]
93. Kraulis PJ. J. Appl. Crystallogr 1991;24:946–950.

Figure 1.

Putative mechanism for the transphosphorylation (A) and hydrolysis (B) reactions catalyzed by RNase A (33). In the hydrolysis reaction, the hydrogen bonds between Asp121...His119...H₂O resemble those in the renowned catalytic triad of serine proteases.

Figure 2. Structure of the active site of crystalline D121N RNase A (A) and D121A RNase A (B). Structures were determined by X-ray diffraction analysis to a resolution of 1.6 Å. Hydrogen bonds of ≤ 3.2 Å are labeled. Enzymes were crystallized at pH 5.2 (D121N RNase A) or pH 5.3 (D121A RNase A).

Figure 3.

Structural differences between D121N RNase A and D121A RNase A and their semisynthetic analogs. (A) Stereoview of the overlap of the active sites of D121N RNase A (thick red lines) and the semisynthetic D121N analog [thin blue lines; (30)]. The sD121N analog contains a sulfate ion in its active site. (B) Stereoview of the overlap of the active sites of D121A RNase A (thick red lines) and the sD121A analog [thin blue lines; (30)]. D121A RNase A contains an acetate ion in its active site, and the sD121A analog contains a sulfate ion in its active site. Water molecules are depicted for the variants (red closed spheres) and semisynthetic analogs (blue open spheres). This figure was created with the program MOLSCRIPT (93).

Figure 4.

Plot of $\log(k_{\text{cat}}/K_m)$ vs pH for the hydrolysis of cUMP by wild-type RNase A (E), D121N RNase A (I), and D121A RNase A (D). The curves are nonlinear least-squares fits of the data to eq 1. To simplify comparisons, the values of k_{cat}/K_m for D121N RNase A and D121A RNase A were multiplied by scaling factors of 2.2. and 13, respectively.

Figure 5. Plot of $\log(k_{\text{cat}})$ vs pH for the hydrolysis of cUMP by wild-type RNase A (E), D121N RNase A (I), and D121A RNase A (D). The curves are nonlinear least-squares fits of the data to eq 2. To simplify comparisons, the values of k_{cat} for D121N RNase A and D121A RNase A were multiplied by scaling factors of 1.6 and 16, respectively.

Figure 6.

Plot of $-\log(K_p)$ vs pH for the hydrolysis of cUMP by wild-type RNase A (E), D121N RNase A (I), and D121A RNase A (D). The curves are nonlinear least-squares fits of the data to eq 3. No scaling factors were applied to these data.

Table 1**X-Ray Diffraction Analysis Statistics**

Crystal data	D121N RNase A	D121A RNase A
Space group	P2 ₁	P3 ₂ 21
Cell dimensions (Å)	<i>a</i> = 30.52 (1) <i>b</i> = 38.40 (1) <i>c</i> = 53.31 (1) β = 105.7°(1)	<i>a</i> = 64.70 (1) <i>b</i> = 64.70 (1) <i>c</i> = 65.03 (2)
Protein molecules/unit cell	2	6
Data collection statistics		
Resolution (Å)	1.6	1.6
No. of measured reflections	28480	56287
No. of unique reflections	15756	27087
Completeness of data (%)	89	91
$R_{\text{merge}} (I/\sigma > 0.33)^I$	0.019	0.029
Average I/σ (1.7 Å – 1.6 Å)	4.5	1.9
R_{merge} (1.7 Å – 1.6 Å)	0.055	0.200
Final refinement statistics		
RNase A atoms	954	951
Solvent atoms	102	103
R -factor (30.0 Å – 1.6 Å) ²	0.177	0.180
RMS deviation from ideal geometry		
Bond distances (Å)	0.01	0.01
Bond angles (°)	2.1	2.7
Average B -factors (Å ²)		
Protein (mainchain)	16.5	23.8
Protein (all atoms)	22.1	28.4
Acetate		29.7
Solvent	39.0	41.4

$I R_{\text{merge}} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$, where I = observed intensity and $\langle I \rangle$ = averaged intensity obtained from multiple observations of symmetry related reflections.

${}_2R = \sum_{hkl} |F_o - F_c| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

Table 2Steady-State Kinetic Parameters for Catalysis by Wild-Type RNase A, D121N RNase A, and D121A RNase A ^a

Substrate	RNase A	k_{cat} (s ⁻¹)	%	K_m (mM)	%	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	%
UpA	Wild-Type	890 ± 30	100	0.38 ± 0.03	100	2300 ± 190	100
	D121N	190 ± 20	21	1.6 ± 0.2	420	120 ± 20	5
	D121A	30 ± 3	3.4	2.2 ± 0.3	580	14 ± 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	71	510 ± 60	15
	D121A	8.2 ± 0.3	1.7	0.20 ± 0.02	140	40 ± 4	1.2
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	120	0.58 ± 0.10	36
	D121A	0.187 ± 0.006	5.1	1.6 ± 0.2	70	0.11 ± 0.01	7

^aData were obtained at 25 °C in 0.10 M MES buffer, pH 6.0, containing NaCl (to $I = 0.10$ M).

Table 3

pH-Dependencies of the Steady-State Kinetic Parameters for the Hydrolysis of cUMP by Wild-Type RNase A, D121N RNase A, and D121A RNase A

pH ^a	Wild-Type RNase A				D121N RNase A				D121A RNase A				$\Delta\epsilon_{282}$ (M ⁻¹ cm ⁻¹)	$\Delta\epsilon_{293}$ (M ⁻¹ cm ⁻¹)
	k_{cat} (s ⁻¹)	K_m (mM)	K_p (mM)	k_{cat} (s ⁻¹)	K_m (mM)	K_p (mM)	k_{cat} (s ⁻¹)	K_m (mM)	K_p (mM)	k_{cat} (s ⁻¹)	K_m (mM)	K_p (mM)		
3.45 (F)	0.042±0.001	3.79±0.2	2.7	0.029±0.001	5.8±0.6	3.4							958	44.3
4.00 (F)	0.156±0.003	2.16±0.07	0.79	0.096±0.003	3.1±0.2	0.68							995	49.1
4.48 (A)	0.43±0.01	1.45±0.10	0.26	0.216±0.006	2.3±0.2	0.28				0.031±0.001	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				0.055±0.002	0.957±0.007	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				0.090±0.002	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				0.187±0.006	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				0.324±0.004	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				0.169±0.006	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 ^b		19.0	0.0010 ^b		5.0								57.0

^aData were obtained at 25 °C in 0.10 M buffer containing NaCl (to $I = 0.10$ M). Letters in parentheses denote buffer: F = formic acid, A = acetic acid, M = MES, T = Bis-Tris, B = BICINE, P = MOPS, S = AMPSO.

^bRepresents k_{cat}/K_m rather than k_{cat} .

Table 4

Intrinsic Steady-State Kinetic Parameters and Apparent pK_a 's for the Hydrolysis of cUMP by Wild-Type RNase A, D121N RNase A, and D121A RNase A ^a

Parameter	Enzyme	pK_a	pK_b	pK_c	pK_d	k_{cat}/K_{m}^{int} (mM ⁻¹ s ⁻¹)
k_{cat}/K_m	Wild-Type	5.58 ± 0.20	6.01 ± 0.17	3.80 ± 0.12		4.9 ± 1.8
	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
k_{cat}	Wild-Type		$pK_{b,s}$	$pK_{c,s}$		k_{cat}^{int1} (s ⁻¹)
	D121N	6.47 ± 0.14	8.63 ± 0.16	4.99 ± 0.15		10.5 ± 1.0
	D121A	6.32 ± 0.28	7.44 ± 0.15	4.52 ± 0.29		3.9 ± 1.1
K_p	Wild-Type	6.66 ± 0.22	6.95 ± 0.21	4.62 ± 0.75		1.0 ± 0.3
	D121N					
	D121A					
K_p	Wild-Type	$pK_{a,p}$	$pK_{b,p}$	K_p^{int} (mM)		
	D121N	6.12 ± 0.10	8.53 ± 0.05	0.023 ± 0.002		
	D121A	6.03 ± 0.12	7.60 ± 0.10	0.020 ± 0.002		
K_p	Wild-Type	6.00 ± 0.09	7.83 ± 0.04	0.013 ± 0.001		
	D121N					
	D121A					

^aValues were calculated by fitting the data in Table 2 to eq 1 – 3.