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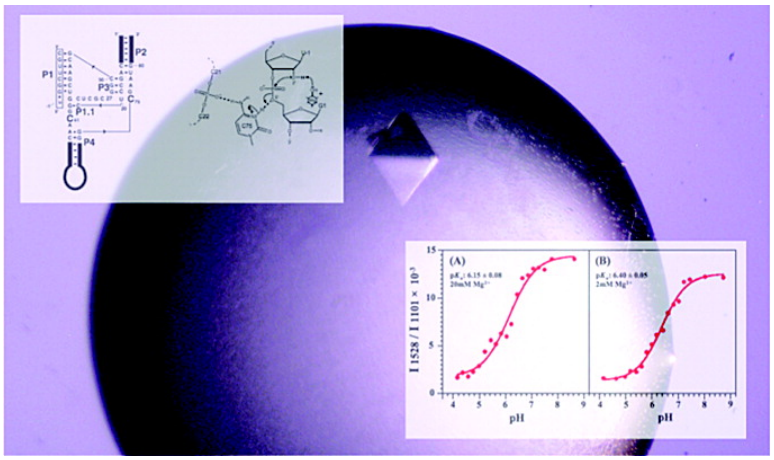

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Direct Measurement of a pK_a near Neutrality for the Catalytic Cytosine in the Genomic HDV Ribozyme Using Raman Crystallography

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Abstract: The hepatitis delta virus (HDV) ribozyme uses a cytosine to facilitate general acid−base catalysis. Biochemical studies suggest that C75 has a pK_a perturbed to near neutrality. To measure this pK_a directly, Raman spectra were recorded on single ribozyme crystals using a Raman microscope. A spectral feature arising from a single neutral cytosine was identified at 1528 cm^{-1}. At low pH, this mode was replaced with a new spectral feature. Monitoring these features as a function of pH revealed pK_a values for the cytosine that couple anticooperatively with Mg^{2+} binding, with values of 6.15 and 6.40 in the presence of 20 and 2 mM Mg^{2+}, respectively. These pK_a values agree well with those obtained from ribozyme activity experiments in solution. To correlate the observed pK_a with a specific nucleotide, crystals of C75U, which is catalytically inactive, were examined. The Raman difference spectra show that this mutation does not affect the conformation of the ribozyme. However, crystals of C75U did not produce a signal from a protonatable cytosine, providing strong evidence that protonation of C75 is being monitored in the wild-type ribozyme. These studies provide the first direct physical measurement of a pK_a near neutrality for a catalytic residue in a ribozyme and show that ribozymes, like their protein enzyme counterparts, can optimize the pK_a of their side chains for proton transfer.

Introduction

Catalytic RNA molecules, or ribozymes, comprise only four nitrogenous nucleobases, which lack the functional diversity of their amino acid counterparts.[1,2] Such functional uniformity has the potential to severely limit the catalytic versatility of RNA. In particular, A and C residues have relatively low pK_a values near ∼4, while G and U residues have relatively high pK_a values near ∼9. Watson−Crick base pairing stabilizes the uncharged species; therefore, RNA secondary structure formation only serves to shift pK_a values even further from neutrality.[3] Unfolding the RNA can shift the pK_a values of A and C toward neutrality due to the proximity of the negatively charged phosphodiester groups, but the shift is small at only ∼0.2 units.[4] Thus, typical nucleobase pK_a values are unsuited for effective proton transfer, which is optimal near neutrality.[5−7] A key mechanistic question for ribozyme catalysis then is whether ribozymes can perturb pK_a values of certain nucleotides toward neutrality to aid proton transfer.

The hepatitis delta virus (HDV)[8] ribozyme is an 85 nt RNA (Figure 1A) that functions in the life cycle of the human hepatitis delta virus, a 1.7 kb circular satellite of hepatitis B. The virus replicates via a double rolling-circle mechanism in which concatemers are resolved by self-cleavage of the closely related genomic and antigenomic HDV ribozymes.[9] The chemical reaction of the HDV ribozyme involves attack on the scissile phosphate by the 2′-OH of U-1 to leave termini of a 2′,3′-cyclic phosphate on U-1 and a 5′-OH on G1 (Figure 1B). The ribozyme uses a general acid/general base mechanism involving cytosine 75 (C75) in proton transfer.[10,11]

The original evidence implicating C75 in proton transfer came from the crystal structure of the self-cleaved form of the ribozyme, which showed hydrogen bonding between the N3 of C75 and the 5′-OH of G1, the leaving group in the reaction.[12] Been and co-workers demonstrated that catalytically inactive

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(8) Abbreviations used: HDV, hepatitis delta virus; CMP, cytidine 5′-monophosphate; C75 is numbered as C76 in the antigenomic version of the ribozyme; C75U, ribozyme variant in which C75 has been mutated to U; MPD, 2-methyl-2,4-pentanediol.

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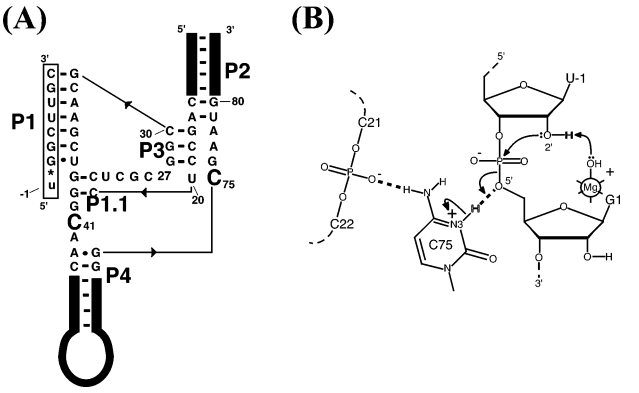

Figure 1. Secondary structure and cleavage mechanism of the HDV ribozyme. (A) Nested double pseudoknot secondary structure of the HDV ribozyme, as determined from crystallography.[12,18,] Numbering is provided every 10 nt, and the direction of chain connectivity is denoted with arrowheads embedded in the lines. The 5 base-paired regions are labeled P1−P4 and P1.1. The cleavage site between U-1 and G+1 is denoted with an asterisk. C41 and C75, which are important to the mechanism and can be protonated, are numbered individually. The sequence used is the fast-reacting G11C sequence used in this study.[11] (B) Possible cleavage mechanism of the HDV ribozyme involving general acid catalysis by C75.[11,17] The nucleophilic 2′-OH of U-1 is deprotonated by a $[Mg(H_2O)_5OH^-]^+$ ion, while the leaving group 5′-O of G1 is protonated by the cationic N3 of C75. The N4 of C75 is depicted as hydrogen bonding with the phosphate of C22, as revealed by crystal structures of the cleaved ribozyme.[12] Close positioning of the Mg^{2+} ion and the cationic C75 is a possible source of the anticooperative interactions between these two species.[11]

C75U or C75 deletion ribozymes could be rescued by addition of imidazole, consistent with C75 serving a histidine-like role.[10] Subsequent studies showed that the observed rate of the reaction depends on the pK_a of the rescuing small molecule in a log-linear fashion, providing strong evidence in support of a role for C75 in proton transfer in addition to any role in electrostatic stabilization.[13,14]

If C75 is involved in proton transfer, then the question arises as to whether it acts as the general acid or general base. By invoking the principle of microscopic reversibility, the crystal structure of the self-cleaved ribozyme implicated C75 as the general acid in the cleavage reaction, and this hypothesis has been supported by a number of biochemical experiments.[11,15−17] However, a crystal structure of the substrate-bound form of the ribozyme, in which the ribozyme was inactivated by a C75U mutation, shows a hydrated Mg^{2+} ion at the 5′-O of G1, and C75U could be moved near the 2′-OH of U-1 by modeling.[18] In contrast to biochemical experiments, this structural model implicates C75 as the general base in the cleavage reaction. Although there is not a consensus on the precise role for C75 in the mechanism, there is solid agreement that C75 is involved in proton transfer and that shifting of the pK_a toward neutrality would be beneficial to function.

The pK_a of C75, as determined on the basis of kinetic studies, is near neutrality. Moreover, kinetics experiments to assess ribozyme activity in solution show that proton binding at C75 is coupled anticooperatively with Mg^{2+} binding.[11,16] This coupling has been attributed to electrostatic repulsion between a closely positioned protonated cytosine and a magnesium ion. Although rate−pH profiles are helpful in determining pK_a values of enzyme residues, they are notoriously difficult to interpret. Observed pK_a values can be complicated by changes in the rate-limiting step of the reaction to pH-independent conformational changes,[19] contributions of pH-independent reaction channels,[15] and acid and base denaturation of the enzyme.[4,20] Although numerous controls have been carried out on the HDV ribozyme to address these issues, a direct measurement of the pK_a of C75 would provide significantly stronger support for this mechanism.

Here we use a Raman microscope to elucidate detailed chemical changes in single crystals of the HDV ribozyme. This approach has been dubbed "Raman crystallography" and has been used to probe chemical changes due to ligand binding or chemical reactions in protein crystals.[21] The present work is the first application to ribozyme crystals. For any macromolecule, the effectiveness of the Raman crystallographic method is due to two major factors. First, the macromolecule is present in the crystal in tens of millimolar concentrations. This is about 2 orders of magnitude greater than can be attained in solution and leads to commensurately higher intensities of Raman signals. Second, interference from a broad "light background", due to weak luminescence or sample aggregation for solution samples, is essentially absent for most crystal samples. We show that Raman crystallography can probe the effects of pH change in crystals of the HDV ribozyme, even at the level of an individual cytosine ring. Experiments on crystals of the C75U HDV ribozyme variant support assignment of the individual cytosine being probed in the wild-type ribozyme to the active-site C75.

Experimental Section

Chemicals. Cytidine 5′-monophosphate (CMP), 2-methyl-2,4-pentanediol (MPD), spermine, and salts were purchased from Sigma Chemical and used without further purification. Milli Q ultrapurified deionized water was used for sample preparation. CMP was dissolved in 50 mM acetate or cacodylate buffer at a final concentration of 90 mM. Acetate buffer was used for the Raman spectra obtained below pH 5.4 and cacodylate buffer for those above pH 5.4.

Preparation of Wild-Type and C75U Ribozymes. The HDV ribozyme was designed on the basis of fast-folding ribozymes previously characterized.[22−24] Briefly, the construct consisted of two strands. The longer strand begins at the 5′-end of P2 and ends at its 3′-end (Figure 1A). This strand was made by T7 transcription and purified on a denaturing polyacrylamide gel. The shorter strand, referred to as the "inhibitor strand" since the reactive 2′-OH has been substituted, is from −1 to the 3′-end of P1 and was prepared by solid-phase chemical synthesis (Dharmacon). This strand contained a 2′-H, -F, or -OCH_3 at the −1 nucleotide to prevent reaction in the presence of the annealed ribozyme and Mg^{2+} ions. The C75U mutant was prepared by mutagenic PCR, and the sequence was confirmed by dideoxy sequencing, which was carried out after both minipreps and maxipreps to control for sequence reversions.

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Raman Spectroscopy of Aqueous CMP Samples. For CMP in solution, Raman spectra were acquired using 647.1 nm Kr^+ laser excitation from an Innova 400 krypton laser (Coherent, Inc.), a back-illuminated charged-coupled device (CCD) detector (Princeton Instruments, Inc.) operating at 183 K, and a Holospec f/1.4 axial transmission spectrometer (Kaiser Optical Systems, Inc.) employed as a single monochromator, as described previously.[25] The CMP sample, which was studied in solution, was held in a cuvette (50 μL) under the 90° excitation/collection geometry, and data were collected using laser power of ~1000 mW with spectral acquisition times of 5 min. The CMP samples were recorded from pH 3.0 to 7.0 with an interval of 0.2 units. Wavenumber calibration was performed by recording the Raman spectrum of cyclohexanone, which yields Raman band positions to within ± 1 cm^{-1}. The Raman spectrum of the buffer at a given pH was subtracted from that of the CMP solution with the same pH, which gives the spectrum of the CMP at the corresponding pH value. The subtraction of data sets and data analysis were performed using GRAMS/32 software (Galactic Industries, Inc.).

Crystal Preparation. Crystals of the highly reactive variant of the HDV ribozyme[24] were prepared using the hanging drop vapor diffusion method (unpublished results). Briefly, equimolar concentrations of HDV ribozyme and inhibitor strands were mixed and incubated at 50 °C for 10 min in a buffer containing 10 mM cacodylate (pH 6.5) and 5 mM $MgCl_2$. Cubic crystals with dimensions of 0.35 mm × 0.35 mm × 0.35 mm were obtained using 35% MPD, 10 mM $MgCl_2$, 0.5 mM spermine, and cacodylate (pH 6.5) buffer as a precipitant. Crystals grew in 1−3 weeks, belong to the space group *I*4(1)32, and diffract synchrotron X-rays to ~4.2 Å (unpublished results). Crystals of C75U variant were obtained under similar conditions. HDV ribozyme crystals were held in a stabilization buffer containing 20 mM $MgCl_2$, 2 mM spermine, and 50% MPD (v/v) in addition to acetate or cacodylate buffer (50 mM) at a variety of pH values.

Raman Spectroscopy of HDV Ribozyme Single Crystals. Raman spectra of single HDV ribozyme crystals were acquired using a HoloLab Series 5000 Raman microscope (Kaiser Optical Systems). In these experiments, the 100 mW of 647.1 nm laser excitation was injected into a single crystal in a hanging drop via the microscope's HoloProbe and objective lens.[26] The focusing process can be monitored by a video camera incorporated into the microscope. The back-scattered light from the crystal was collected by using the long focal-length ×50 microscope objective lens for 100 s. Before acquiring crystal Raman data, the Raman microscope was calibrated by using standard neon and tungsten lamps, which gives the Raman band positions within ± 1 cm^{-1}. In order to collect data from the same crystal at different pH conditions, crystal stabilization solutions were prepared with a series of acetate or cacodylate buffers differing by 0.2 units. These were soaked into the crystal one-by-one by changing the surrounding artificial mother liquor. After each change, Raman data were collected from the crystal and the surrounding buffer, separately. In multiple experiments, we found equilibration after 15−20 min with no further change after that time. The HDV ribozyme crystal Raman spectrum was obtained by subtracting the buffer spectrum from that of the HDV ribozyme crystal containing buffer.

The intensity of Raman scattering from a crystal can depend on the relative orientation of the laser beam and the crystal.[27] However, in the present case, the laser beam is not polarized and the highly symmetric nature of the cubic space group gives rise to an essentially isotropic condition in which there is no dependence of scattering intensity on crystal orientation. This is borne out by reproducibility of independent experiments we have performed on more than 50 crystals in multiple orientations.

Quantum Mechanical Computation. In order to analyze the vibrational modes of cytosine in CMP, cytosine with a methyl group instead of ribose at its N1 site (1-MeC) was used as a simple model compound to carry out quantum mechanical computation by using Gaussian 98 software,[28] as described previously.[29] Neutral and N3-protonated forms of 1-MeC were analyzed by this method.

Curve Fitting of Cytosine Raman Bands. Band fitting of cytosine Raman data was performed with the CurveFit.Ab routine of GRAMS/32, which is based on the Levenberg−Marquardt nonlinear least-squares method.[30] A mixed Gaussian and Lorentzian peak shape was applied in the curve fitting process. Two Raman bands, centered at ~1532 and ~1547 cm^{-1} for CMP and ~1528 and ~1536 cm^{-1} for HDV ribozyme, were fitted as shown in Figures 2 and 5. The fitting quality can be assessed by comparing the χ^2 of the fits.

Determination of pK_a of Cytosine in CMP and HDV Crystal. The pK_a values of cytosine in CMP and HDV ribozyme crystals were determined from the change in the intensity of the Raman marker band (I_{obs}) with pH according to a two-state model using the equation

$$I_{obs} = I_R + \frac{I_{RH^+} - I_R}{1 + 10^{pH-pK_a}} \quad (1)$$

where I_{RH^+} and I_R represent the intensity of the Raman marker band of protonated and deprotonated cytosine, respectively. This model assumed a single protonation was involved in switching between the two states, which gave good fits to the data. In the present study, an internal Raman intensity standard band was used to normalize the Raman marker band of cytosine. In this case, eq 1 becomes

$$I_{obs}/I_{I,S} = I_R/I_{I,S} + \frac{I_{RH^+}/I_{I,S} - I_R/I_{I,S}}{1 + 10^{pH-pK_a}} \quad (2)$$

where $I_{I,S}$ is the intensity of the internal standard band, which is insensitive to pH titration. For both CMP in solution and HDV ribozyme crystals, we used the PO_2^- symmetric stretch as the internal standard. Although the pK_a of the phosphate of CMP is ~6, this band is insensitive to pH in the ranges used in this study (not shown). Equation 2 helps ensure that the observed intensity change of the cytosine Raman marker band is due to pH effect rather than concentration discrepancies between samples. Values of pK_as of cytosine in CMP and the HDV ribozyme were determined by fitting the data to eq 2 using KaleidaGraph (Synergy Software). In some cases, the exponent for the second term in the denominator of eq 2 was multiplied by a Hill coefficient, n_{Hill} (see eq 3). However, the value of n_{Hill} was close to unity and so was held constant at 1 for analysis.

$$I_{obs}/I_{I,S} = I_R/I_{I,S} + \frac{I_{RH^+}/I_{I,S} - I_R/I_{I,S}}{1 + 10^{n_{Hill}(pH-pK_a)}} \quad (3)$$

Results

Using Raman Marker Bands to Measure the pK_a of Cytosine in CMP. We studied the pH dependence of a concentrated solution of CMP to establish regions of the spectrum that would allow pK_a determination. This small system is also amenable to quantum calculations used to determine the molecular origin of the pH-sensitive spectral features. The Raman spectrum of 5′-CMP reveals features between 1505 and 1575 cm^{-1} that are characteristic of the cytosine base and are strongly pH-dependent. Curve fitting of spectra obtained at pH 6.0 revealed bands near 1532 and 1547 cm^{-1} (Figure 2). Upon lowering pH from 7.0 to 3.0, the band at 1532 cm^{-1} disappeared.

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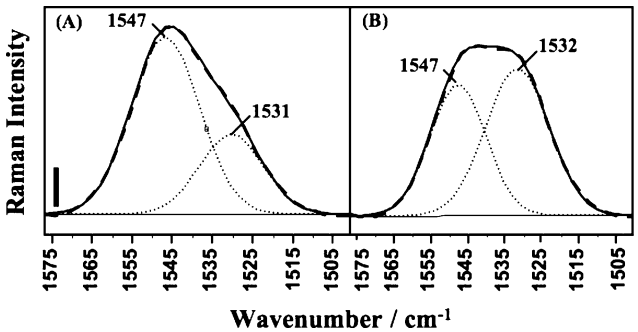

Figure 2. Curve fitting of the cytosine band of CMP in the spectral region of 1575−1505 cm^{-1}. Spectra were obtained at different pH values: (A) pH 4.0 and (B) pH 6.0. The component band at ∼1532 cm^{-1} is the Raman marker for neutral cytosine in CMP. The dashed lines are the experimentally determined spectra. The dotted lines denote individual component functions, and the solid lines indicate the sum of the functions. The peak positions are labeled. The vertical bar represents a 5000 photon event, and the *y*-axis scales linearly with the number of photons.

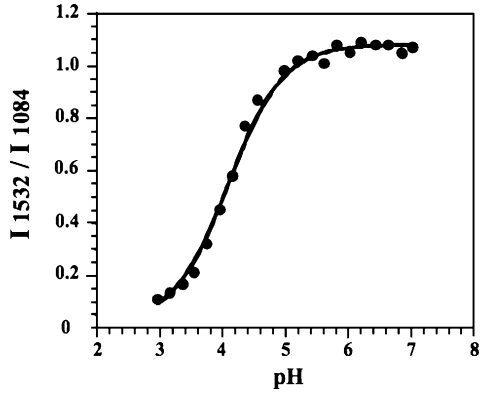

Figure 3. The pK_a measurement of cytosine in CMP. The *y*-axis is the relative intensity ratio of the 1532 cm^{-1} band (neutral cytosine) to the 1084 cm^{-1} band (PO_2^-, internal standard). A pK_a of 4.09 ± 0.04 was obtained using eq 2.

Density functional theory calculations were carried out on 1-MeC, in which the methyl group mimics the glycosidic linkage and allows protonation at N3 but not N1.[31,32] These calculations, which were at the B3LYP/6-31+G(d) level, give tentative assignments of the two bands. For neutral cytosine, the 1547 cm^{-1} band is predominantly due to NH_2 bending, while the 1532 cm^{-1} band is mainly caused by N3═C4 stretching. Upon protonation of N3, the N3═C4 stretching mode is replaced by an in-plane N3−H stretching coupled with ring deformation and thus the 1532 cm^{-1} band disappears. The new vibrational mode shows a frequency of ∼1546 cm^{-1}, which shares the same spectral region with NH_2 bending of neutral cytosine. Because the intensity of the 1532 cm^{-1} band reflects the population of neutral cytosine in CMP, the integrated area of the band can be used as a Raman marker to measure directly the pK_a of cytosine in CMP. Benevides et al. have also noted that a similar feature can be used to monitor cytosine protonation/deprotonation in deoxynucleic acids.[33]

In implementing eq 2, the Raman band at 1084 cm^{-1} was used as an internal standard. This band represents PO_2^-

Table 1. Comparison of pK_a Values from Raman and Biochemical Studies[a]

condition	CMP	C75[b] (2 mM Mg^{2+})	C75[b] (20 mM Mg^{2+})
Raman (Hill = 1)[c]	4.09 ± 0.04[d]	6.40 ± 0.05	6.15 ± 0.08[e]
Raman[f]	4.13 ± 0.02[d]	6.40 ± 0.05	6.14 ± 0.09
(n_{Hill})	(1.3 ± 0.07)[g]	(0.93 ± 0.09)	(0.86 ± 0.15)
biochemical[h]	4.30[i]	6.5[j]	6.1[k]

[a] All Raman studies are in single crystals of the genomic HDV ribozyme except where noted. [b] Values are for the 2′-methoxy substitution. [c] These data were fit with the Hill coefficient forced to 1. [d] Raman experiments on CMP were in solution (50 mM acetate or cacodylate buffer using 90 mM CMP). [e] A pK_a value of 6.19 ± 0.06 was obtained for the 2′-deoxy substitution, and a pK_a of 6.08 ± 0.11 was obtained for the 2′-fluoro substitution. [f] These data were fit with a Hill coefficient, n_{Hill}, allowed to vary, which is provided directly below the pK_a. [g] The lower pH baseline was poorly defined, which caused difficulty in determining the Hill constant. [h] Biochemical studies are on the HDV ribozyme as inferred from kinetics measurements in solution, except where noted. [i] Measured spectrophotometrically in 25 mM sodium phosphate, which is similar to conditions used in Raman CMP experiments.[35] [j] Value measured in 1.9 mM Mg^{2+}.[11] [k] Value measured in 10 mM Mg^{2+}; it can be noted that 10 and 50 mM Mg^{2+} gave nearly identical pK_a values in solution of 6.1 and 5.8, respectively.[11]

symmetrical stretching.[27] Although the phosphomonoester group of CMP has two ionizable protons with typical pK_a values of 1.0 and 6.2,[34,35] we did not observe any PO_2^- intensity in the difference spectrum [CMP pH 7.0] minus [CMP pH 3.0] (not shown). Thus, within the present pH range of 3.0−7.0 for CMP, the 1084 cm^{-1} band is insensitive to pH change; its intensity only reflects the sample concentration and can be used as an internal standard. The ratio of the intensities of 1532/1084 cm^{-1} was measured as a function of pH and fitted to eq 2. Figure 3 shows the pK_a fitting curve of cytosine in CMP and gives a pK_a of 4.09 ± 0.04 (Table 1). The same strategy was used to measure the pK_a of a cytosine residue in an HDV ribozyme crystal. To further demonstrate that the phosphate group pK_a of 6.2 does not interfere with curve fitting, the absolute value of the intensity of the 1532 cm^{-1} band was also plotted as a function of pH. This revealed a pK_a of 4.12 ± 0.03 with a Hill coefficient of 1.36 or 4.07 ± 0.04 when setting the Hill coefficient = 1 (not shown). These numbers are identical within error to the value obtained from plotting the 1532/1084 ratio.

Using Raman Marker Band to Quantitate Non-Base-Paired Cytosines in an HDV Ribozyme Crystal. A Raman spectrum of a single HDV ribozyme crystal is shown in Figure 4A. This crystal is of a fast-reacting version of the ribozyme, which contains a cytosine at position 75 and is inhibited by substituting the 2′-OH at the −1 nucleotide by 2′-OCH_3. In other instances, the 2′-OH was substituted with 2′-H or 2′-F (see below). Due to several decades of research into the vibrational spectra of nucleic acids, the origins of the most intense modes are known, and the assignments are given in Table 2.[27,36,37] As seen in Figure 4A, the data are of high quality, superior to those that can be obtained from samples in solution, and have a signal-to-noise ratio of about 300. The need for high-quality data is apparent since non-base-paired cytosine produces the weak band near 1530 cm^{-1}. The quality of signal arises in large part from

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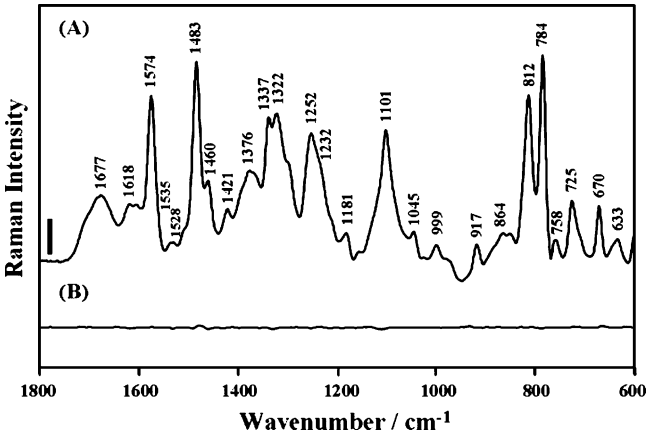

Figure 4. Raman spectra of HDV ribozyme crystals. (A) The spectrum of the wild-type ribozyme (using 2′-OCH$_3$ substitution) in the region of 1800−600 cm^{-1}. The crystal was surrounded by cacodylate buffer containing 20 mM Mg^{2+} at pH 7.0 at room temperature (22 °C). Additional experimental details are provided in the Experimental Section. The frequencies of major Raman bands are marked in the spectrum, and assignments of these bands are shown in Table 2. The vertical bar represents a 5000 photon event, and the *y*-axis scales linearly with the number of photons. (B) Raman difference spectrum between C75U and wild-type HDV ribozymes (both using the 2′-H substitution) shows that the conformations of the RNA molecules in C75U and wild-type are very similar. The HDV ribozyme crystal was surrounded by cacodylate buffer containing 20 mM Mg^{2+} at pH 7.0 at room temperature (22 °C), conditions wherein C75 in the wild-type ribozyme is largely deprotonated.

Table 2. Wavenumbers (cm^{-1}) and Assignment of Major Raman Bands Observed in Raman Spectrum of HDV Ribozyme Crystal[a]

wavenumber	assignment
1677 (m)	base C=O
1574 (s)	A, G
1535 (w)	C
1528 (w)	C
1483 (vs)	G, A
1460 (w)	C−H (ring)
1421 (w)	A, G
1376 (m)	A, G
1337 (s)	A
1322 (s)	G
1252 (s)	C
1232 (sh)	U
1181 (w)	G
1101 (s)	PO_2^-
1045 (w)	ribose
999 (w)	ribose
917 (w)	ribose
812 (s)	O−P−O
784 (vs)	C, U
725 (m)	A
670 (m)	G
633 (w)	G

[a] Vs, very strong; s, strong; m, medium; w, weak; sh, shoulder. The features assigned to the bases are due to ring modes.

use of crystals, which minimizes conformational heterogeneity and maximizes the concentration of RNA being sampled. Because this region of the spectrum corresponds to the modes seen in Figure 2 for CMP, and it occurs in a spectral window uncluttered by other RNA features, it provides an excellent opportunity for pK_a measurement.

Band fitting was undertaken for the HDV ribozyme profile near 1530 cm^{-1} (Figure 5). The profile can be successfully fitted by two peaks near 1536 and 1528 cm^{-1} that correspond to the two bands seen in CMP near 1547 and 1532 cm^{-1} in Figure 2. The small shifts between the peak positions in the HDV

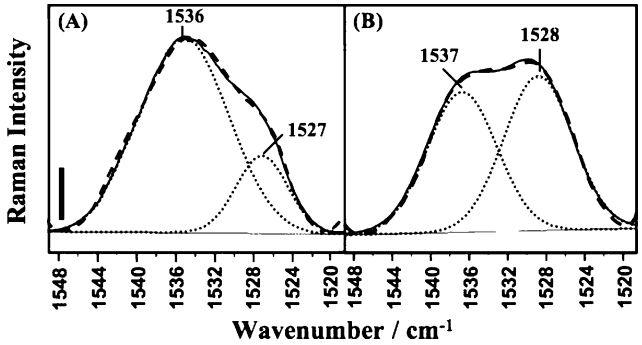

Figure 5. Curve fitting of cytosine band of the HDV ribozyme crystals. Spectra of a HDV ribozyme crystal (using 2′-OCH$_3$ substitution) in the spectral region of 1548−1520 cm^{-1} were obtained at different pH values: (A) pH 5.0 and (B) pH 7.2. The component band at ∼1528 cm^{-1} is the Raman marker for neutral cytosine in HDV ribozyme crystal. The dashed lines are the experimentally determined spectra. The dotted lines denote individual component functions, and the solid lines indicate the sum of the functions. The peak positions are labeled. The vertical bar represents a 500 photon event, and the *y*-axis scales linearly with the number of photons.

ribozyme and CMP profiles are likely due to environmental factors affecting the cytosine modes for the ring within the HDV ribozyme.

For CMP, a linear relationship was found between the intensity of the neutral cytosine mode near 1532 cm^{-1} and CMP concentration (Figure S1). These data were collected at pH 7.0, which is well above the pK_a for the N3 of CMP (Table 1), assuring that neutral cytosine is being monitored. In an HDV ribozyme crystal, at pH 7.0 and 20 mM Mg^{2+}, which is ∼1 unit above the observed pK_a (Table 1), the measured integrated intensity of the band near 1528 cm^{-1} (the counterpart of the 1532 cm^{-1} feature in CMP) corresponds to an uncorrected concentration of neutral cytosine of 24 mM in the crystal (Figure S1). When corrected for the ∼10% of the ribozyme still protonated at this pH, the concentration of ionizable cytosine in the crystal is ∼27 mM. The concentration of HDV ribozyme molecules in the crystal is calculated to be 26 mM,[38] in good agreement with the concentration of ionizable cytosine measured by Raman. Thus, there appears to be a single ionizable cytosine per HDV ribozyme in the crystal. Other non-base-paired and base-paired cytosines also contribute intensity in the 1530 cm^{-1} region (Gong, Christian, and Carey, unpublished work). However, the Raman intensity from these does not change in the pH range of 4.2−8.8 (see C75U below). Thus, we are measuring the pH-dependent change in the Raman signal from only one cytosine on a constant background.

Measuring a Cytosine pK_a in an HDV Ribozyme Crystal. As described, the 1528 cm^{-1} feature can be assigned to a single neutral cytosine in the HDV ribozyme and, based on mutagenesis (see below), assigned to C75. These characteristics allow us to measure the pK_a value for this ribozyme cytosine. The crystals used in this experiment contained the 2′-OCH$_3$ substitution at the U-1 position of the inhibitor strand. Ratios of the area of the fitted band, centered at 1528 cm^{-1}, and that of the PO_2^- band, centered at 1101 cm^{-1}, were determined as a function of pH for two concentrations of Mg^{2+}, 20 and 2 mM

(38) Examination of the Matthews coefficient of the crystals suggests that there are 2 molecules in each of the 48 asymmetric units within the unit cell or 1.59×10^{-22} mol HDV ribozyme per unit cell. The volume of the unit cell is (183 Å)^3 or 6.12×10^{-21} L. The concentration of HDV ribozyme in the crystal is therefore calculated to be 26 mM.

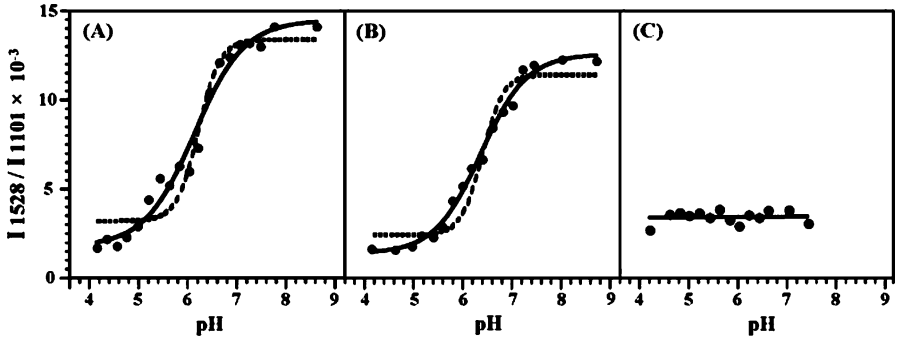

Figure 6. The pH titration of HDV ribozyme crystals. The pK_as of cytosine in an HDV ribozyme crystal (using the 2′-OCH_3 substitution) containing 20 and 2 mM Mg^{2+} were fit using eq 2 as shown by the solid line. For comparison, the best fit to eq 3 using a Hill constant of 2 is shown with a dashed line. The model with a Hill constant of 1 provides the best description of the observed data. The *y*-axis is the relative intensity ratio of 1528 cm^{-1} band (neutral cytosine) to the intensity of the band at 1101 cm^{-1} (PO_2^-, internal standard). A pK_a of 6.15 ± 0.08 at 20 mM Mg^{2+} (A) and a pK_a of 6.40 ± 0.05 at 2 mM Mg^{2+} (B) were obtained, demonstrating that the observed protonation of cytosine in an HDV ribozyme crystal is anticooperative with magnesium binding. The magnitude and the anticooperative coupling between the pK_a and magnesium concentration is in agreement with values measured for the HDV ribozyme in dilute solution by kinetics methods (Table 1). (C) The Raman spectra of a C75U mutant ribozyme crystal (using 2′-H substitution) was examined in the pH range of 4.2−7.4. No signal from a protonatable cytosine was observed, supporting the assignment of the titratable group in wild-type HDV to C75.

(Figure 6A,B). At each Mg^{2+} concentration, the data could be fit to a single pK_a, consistent with our model that the 1528 cm^{-1} feature arises from a single cytosine. At 20 mM Mg^{2+}, a pK_a of 6.15 ± 0.08 was obtained, which increased to 6.40 ± 0.05 at 2 mM Mg^{2+} (Table 1). The increase in pK_a with decreasing Mg^{2+} concentration represents anticooperative coupling between proton and magnesium binding. These pK_a values and the anticooperative ion binding are similar to those observed in solution kinetics experiments.[11,39] For example, pK_a values of 5.8, 6.1, and 6.5 were found at Mg^{2+} values of 50, 10, and 1.9 mM Mg^{2+}, respectively.[11] Titrations were reproducible, and crystals showed no signs of cracking or hysteresis.

To test whether the nature of the substitution at the 2′-position of the inhibitor strand affected the conformation of the active site, tandem experiments were also performed with 2′-H and 2′-F modifications in 20 mM Mg^{2+}. The pK_a values for the 2′-H and 2′-F substitutions were 6.19 ± 0.06 and 6.08 ± 0.11, which were identical within experimental error to the value of 6.15 ± 0.08 for the 2′-OCH_3 substitution (Table 1). Absence of significant differences in the pK_a values for the 2′-H, 2′-F, and 2′-OCH_3 substitutions suggests that all three substitutions are relatively nonperturbing to the active site structure.

The C75U Mutation Abolishes Cytosine Protonation. Since C75 is an active-site residue known to be crucial for catalysis, it is a strong candidate for the cytosine that is undergoing titration near pH 6 (Figure 6A,B). In an effort to correlate the observed pK_a with C75, the inactive C75U variant of the HDV ribozyme was prepared and crystallized. A difference Raman spectrum calculated using C75U and wild-type ribozyme crystals shows that the C75U and wild-type HDV ribozymes are in similar conformations under conditions where C75 is largely deprotonated (Figure 4B). The difference spectrum exhibits only minor features that have about 1% of the intensity found in the parent spectra. In particular, we see almost no Raman signals near 813 cm^{-1}, which is in the C−O−P−O−C stretching region. Since this is very sensitive to conformational change, the lack of this signal demonstrates that little overall conformational change occurs upon C75U substitution. This is in agreement with X-ray crystallographic data of Ke and coworkers, which revealed only minor conformational differences between the wild-type and C75U mutant.[18]

As can be seen in Figure 6C for a C75U HDV ribozyme crystal, the intensity ratio I_{1528}/I_{1101} is constant throughout the pH 4.2−7.4 range, showing no evidence of a titration. Since the C41 base triple is intact in the C75U crystal structure[18] and because the only significant differences in this structure are localized near residue 75,[18] the data in Figure 6C support assignment of the titratable group in wild-type HDV ribozyme (Figure 6A,B) to C75.

Discussion

Raman Spectroscopy is Uniquely Applicable to RNA Enzymes. In the present work, Raman spectroscopy has been applied to determine pK_a values in a functional RNA. In addition, Raman offers several advantages for other mechanistic studies of RNA enzymes, and some of these are unique. Herein, we are discussing nonresonance ("normal") Raman spectroscopy which does not utilize a chromophore and is widely applicable.[27] Raman can follow chemical events, including reactions in single crystals and inform the crystallographer of the optimal conditions for trapping target species for X-ray analysis.[21] In favorable cases, a direct comparison can be made between the same processes occurring in crystals and in solution.[26] The intrinsic time scale of the Raman effect is subpicosecond, thus the Raman spectrum is essentially an instantaneous snapshot of all the molecules giving rise to the spectrum, and spectral line broadening due to chemical exchange events is not seen. By selective isotopic labeling and using Raman difference spectroscopy, it should be possible to obtain the vibrational spectrum of individual groups in RNA molecules to probe the very groups, or bonds, that are bringing about catalysis or undergoing chemical transformation. Finally, Raman offers a unique probe for the RNA−metal interactions that play vital roles in RNA folding, structure, and catalysis (unpublished work from our laboratories and E. Christian and M. Harris).

Does the HDV Ribozyme Adopt a Catalytically Competent Conformation in the Crystalline State? The HDV ribozyme catalyzes an RNA cleavage reaction using general acid−base catalysis. To dissect the role of C75 in catalysis, we set out to

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measure the pK_a of this nucleobase directly. It is desirable to study the precleaved state of a ribozyme for a number of reasons. This state contains the scissile phosphate and so has the greatest potential to provide insight into the chemical step; moreover, the scissile phosphate may provide the electrostatic background necessary for full pK_a shifting.[4,15,40] However, studying such a state requires that the chemical step be inhibited in some way. Most of the previous crystallographic studies on the precleaved HDV ribozyme used the C75U mutation to prevent chemical reaction.[18] This mutant is catalytically inactive.[10] Comparison of the C75U precleaved structure with the wild-type post-cleavage structure revealed an active site that underwent a conformational change.[12,18] The C75U structure was consistent with a hydrated magnesium ion acting as a general acid and C75 as a general base. However, the role of C75 had to be inferred from computational studies, as it was not positioned in either the general acid or base role in the experimental structure.[18,41,42] Another crystal structure of the precleaved state was of wild-type, but without Mg^{2+} ions.[18] However, such ions have profound effects on the fold (manuscript in preparation) and couple to pK_a values (Figure 6), suggesting their presence is essential to functionally relevant results.

In the present study, we left C75 intact and inhibited the reaction by changing the nucleophile 2′-OH of U-1. This change allowed us to grow crystals in the presence of Mg^{2+} ions and a cytosine at position 75. To test for any structural or chemical changes induced by altering the functional group at the 2′-position of U-1, we made a series of modifications in which this hydroxyl group was changed to -H, which removes the ability to accept or donate a hydrogen bond, or -F or -OCH_3, which retain the ability to accept a hydrogen bond. We prepared crystals of the genomic HDV ribozyme and determined pK_a values in single crystals by titrations.

We observed protonation of a single unpaired cytosine base in the ribozyme that appears to be arising from C75. The observed pK_a values were not dependent on the identity of the functional group at the 2′-position of U-1. For example, methoxy, deoxy, and fluoro 2′-substitutions in the presence of 20 mM Mg^{2+} gave essentially identical pK_a values (Table 1). This suggests that the nature of the inhibitor strand does not affect the ribozyme active-site structure.

To determine whether the conformation of the ribozyme in the crystal was relevant to the ribozyme conformation in solution, pK_a values obtained from the present study were compared to solution kinetic experiments. Nearly identical pK_a values are obtained in the crystal and in solution kinetics experiments (Table 1).[11] For example, the observed pK_a was 6.40 in 2 mM Mg^{2+} in the crystal and 6.5 in 1.9 mM Mg^{2+} in solution, while the pK_a of C75 was 6.15 in 20 mM Mg^{2+} in the crystals and 6.1 in 10 mM Mg^{2+} in solution (Table 1). These comparisons suggest that the crystal has proper protonation and Mg^{2+} binding behavior and is therefore in a catalytical competent conformation.

Does the Observed pK_a Correspond to C75? The crystal structure of the HDV ribozyme suggests that there are two potential protonated cytosines in the RNA: C75, in the active site of the ribozyme, and C41, which is involved in a structural interaction.[12] The measured pK_a value of the observed cytosine is near neutrality and couples anticooperatively with Mg^{2+} binding. Solution studies indicate that protonation of C75 exhibits anticooperative coupling with Mg^{2+}, consistent with the Raman data, while protonation of C41 exhibits cooperative coupling with Mg^{2+}.[11,15,16] To further support assignment of the observed pK_a to C75, this nucleotide was changed to a uridine and crystallized. No change in cytosine protonation was observed using the C75U mutant, again suggesting that the pK_a of C75 is being observed.

The simplest interpretation of these results is that the Raman experiment is monitoring the protonation state of C75, and that the pK_a of this nucleobase is near neutrality. The experiments do not absolutely rule out the possibility that the presence of C75 is required for protonation of a different cytosine elsewhere in the ribozyme. However, there are three lines of evidence inconsistent with this interpretation. First, there is no evidence for a large-scale conformational change upon substituting C75 with U. The difference spectra between the wild-type ribozyme and C75U demonstrate little or no conformational rearrangements when C75 is substituted with uridine (Figure 4B). Second, the observed pK_a, if belonging to C41, would be expected to increase with Mg^{2+} concentration, not decrease.[11,15,16] Third, the ability to titrate the ribozyme in the crystal is consistent with relatively minor changes in conformation between the C75-protonated and unprotonated states. No cracking or hysteresis was observed in the crystals, a given crystal could be titrated multiple times, and the titration could go up in pH or down in pH and give similar values. This plasticity suggests that large-scale motions such as docking and undocking of helices are not needed to change between these states of the system. This conclusion is consistent with the very similar overall folds between self-cleaved and C75U precleaved crystal structures,[12,18] as well as the ability of C75U to be rescued by imidazole addition.[10,11,13] In the absence of any conformational rearrangement upon protonation of the ribozyme, it is unclear from the HDV ribozyme crystal structure[12] how the presence of C75 at the active site could be structurally coupled to a protonatable cytosine elsewhere in the molecule.

Comparison with Previous Analyses. Observation of a pK_a for C75 near neutrality is in contrast to conclusions drawn from a previous study designed to measure the pK_a of C75 in the HDV ribozyme. Luptak and co-workers performed NMR experiments on ^{13}C,^{15}N-cytosine labeled ribozymes in an effort to determine the pK_a of C75.[43] These studies focused largely on the 3′-product of ribozyme self-cleavage and revealed a pK_a of 4.1−4.3 in 3 mM $MgCl_2$ and 70 mM LiCl, which is essentially unshifted from the value of C in single-stranded oligonucleotides.[4] Slightly higher pK_a values were measured in lower divalent ion concentrations, but no value larger than 4.7 was measured in the presence of Mg^{2+} ions.[43] Lack of pK_a shifting in the self-cleaved ribozyme may result from the loss of the negatively charged scissile phosphate. Moreover, the hydrogen bond observed from the 5′-hydroxyl of G1 to the N3 of C75 in the product crystal[12] is predicted to stabilize the

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deprotonated cytosine ring and thereby depress the pK_a of C75 relative to free cytosine.

In the substrate-bound ribozyme, the close proximity of the scissile phosphate to the base of C75 could help stabilize the positive charge on a protonated cytosine.[4,15,40] To address this possibility, NMR experiments were also carried out on pre-cleaved versions of the ribozyme.[43] While the quality of the spectra prevented the pK_a of C75 from being measured, the ^{13}C chemical shifts of C75 were similar to those of deprotonated cytosine in the cleaved form of the ribozyme. The simplest interpretation of these results provided by the authors is that the pK_a of C75 is not shifted by the presence of the scissile phosphate (i.e., pK_a < 5). This conclusion is not in agreement with the results presented here nor with kinetic studies that suggested a near-neutral pK_a for C75.[10,11,17]

This comparison raises the issue of why a pK_a near neutral pH could be measured in the crystalline environment, but not by solution-phase NMR. RNA molecules in solution often adopt multiple conformations that can interconvert on various time scales. For example, single molecule studies on the hairpin ribozyme revealed four non-interconverting populations.[44] Structural heterogeneity of the wild-type HDV ribozyme is a well-documented phenomenon; for example, 5 M urea or 10 M formamide greatly stimulates self-cleavage of the wild-type genomic ribozyme.[45] In addition, Ferre-D'Amare et al. used the U-1A protein binding and selectively purified cleaved RNA to favor formation of the correctly folding conformation prior to crystallization.[12] Ke et al. were able to grow crystals of the substrate-bound ribozyme, but generating a homogeneous sample required a complex refolding protocol out of urea.[18]

To achieve a homogeneous population, we exploited two strategies. First, these experiments relied on a crystal lattice that would favor formation of homogeneously folded RNA molecules. Second, the crystals used here were composed of a genomic HDV ribozyme construct that has fast, monophasic kinetics,[22−24] suggesting these RNAs are less prone to folding heterogeneity. Observation that the crystals grew rapidly without a complicated refolding protocol is consistent with a homogeneous native population. Thus, Raman crystallography provides a means to study the properties of molecules that tend to be heterogeneous when examined in bulk solution.

Spectroscopic approaches, like Raman, report on specific chemical functional groups, in contrast to kinetic studies that monitor only product formation. As such, Raman data have much more information in them. In particular, the Raman data not only support the pK_a measured by kinetics studies but also demonstrate that the observed pK_a reflects ionization of the catalytic cytosine rather than other events such as a change in the rate-limiting step[15,19] or acid−base denaturation.[4,20] Last, it can be noted that our findings argue that pK_a shifting occurs in an accessible ground state, as previously suggested,[7] rather than in the transition state or a high-energy, inaccessible ground state. It is essential to load the proton in the ground state so that the ribozyme is in its functional form when it engages in the chemical step of bond making and breaking.[6,46]

Effects of the Scissile Phosphate and Nucleophilic 2′-Hydroxyl on pK_a. Shifting of the pK_a of a nucleobase could be due to formation of structures that are only stable upon protonation or due to favorable electrostatic interactions with nearby charged groups, such as phosphates.[4,15,40] Nonlinear Poisson−Boltzmann (NLPB) calculations suggest that the latter may be particularly important in the case of the HDV ribozyme.[15,40] The active site has a highly negative potential, and positioning of the scissile phosphate near it may sandwich C75 between two negatively charged phosphate groups that would help drive protonation of C75 and pK_a shifting. Comparison of results reported here for the inhibitor-bound ribozyme with the NMR results for the product-bound ribozyme suggests that the presence of the scissile phosphate alone may shift the pK_a of C75 over 2−3 pH units toward neutrality.

While the experiments described herein indicate that C75 has a pK_a near neutrality and is therefore optimized for general acid−base chemistry, they do not directly address whether C75 acts as a general acid or base. Nonetheless, it is interesting to note that protonation of C75 is not sensitive to the hydrogen bonding nature or size of the substituent at the 2′-position of U-1, suggesting that C75 may not interact with that position. These observations do not support C75 being positioned near the 2′-position of U-1 as proposed in the general base model, but full understanding of this possibility will have to await further structural analysis of HDV ribozyme crystals.

Conclusion

Using a Raman microscope, it has been possible to characterize the protonation of a single cytosine in crystals of the genomic HDV ribozyme. The absence of pH dependence of a cytosine Raman band in the C75U variant strongly suggests that we are observing the titration of the active-site residue C75 in the wild-type ribozyme and that its pK_a is shifted toward neutrality. This initial use of Raman microscopy to follow events in an RNA crystal demonstrates the potential of Raman crystallography to study RNA structure and function.

This study represents the first direct measurement of a pK_a near neutrality for a catalytic residue in a ribozyme and suggests that local environments of functional RNAs can significantly perturb pK_a values toward neutrality. Such shifting has functional consequences for ribozymes, as it allows the nucleobases to assume histidine-like catalytic properties.

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Supporting Information Available: The CMP standard concentration curve and the full ref 28. This material is available free of charge via the Internet at http://pubs.acs.org.

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