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Direct pK_a Measurement of the Active-Site Cytosine in a Genomic Hepatitis Delta Virus Ribozyme

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Abstract: Hepatitis delta virus ribozymes have been proposed to perform self-cleavage via a general acid/base mechanism involving an active-site cytosine, based on evidence from both a crystal structure of the cleavage product and kinetic measurements. To determine whether this cytosine (C75) in the genomic ribozyme has an altered pK_a consistent with its role as a general acid or base, we used ^{13}C NMR to determine its microscopic pK_a in the product form of the ribozyme. The measured pK_a is moderately shifted from that of a free nucleoside or a base-paired cytosine and has the same divalent metal ion dependence as the apparent reaction pK_a's measured kinetically. However, under all conditions tested, the microscopic pK_a is lower than the apparent reaction pK_a, supporting a model in which C75 is deprotonated in the product form of the ribozyme at physiological pH. While additional results suggest that the pK_a is not shifted in the reactant state of the ribozyme, these data cannot rule out elevation of the C75 pK_a in an intermediate state of the transesterification reaction.

Introduction

During rolling-circle replication of the hepatitis delta virus (HDV) RNA, genome-encoded self-cleaving ribozymes produce unit length genomic and anti-genomic RNA products (reviewed by Lai[1]). Two structurally homologous ribozymes, one in the positive strand and one in the negative strand of the virus, carry out site-specific strand scission with a minimal requirement for divalent metal ions.[2,3] A crystal structure of the self-cleaved form of the genomic ribozyme revealed a cytosine (C75) residue proximal to the 5′ hydroxyl leaving group of the transesterification reaction.[4] This led to a hypothesis that C75 acts as a general acid or general base in the reaction,[4–6] having a role similar to that of the catalytic histidines in RNase A (see Figure 1 for a general mechanism).

Recent investigation of the reaction kinetics of both the genomic and anti-genomic HDV ribozymes has provided strong evidence that C75 (C76 in the antigenomic ribozyme) is directly involved in the transesterification reaction. While mutation of this cytosine to G or U inactivates both ribozymes, substitution

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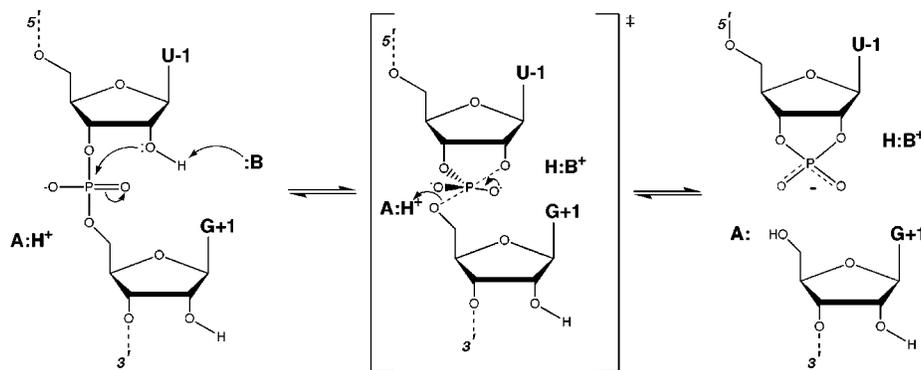(6) Nakano, S.; Chadalavada, D. M.; Bevilacqua, P. C. *Science* **2000**, *287*, 1493−1497.

Figure 1. Proposed mechanism of general acid/base-catalyzed cleavage of a phosphodiester bond in RNA. **B** denotes general base which deprotonates the nucleophile. In a related proposal this base is specific.[8] **A** denotes the general acid which protonates the 5′ leaving group. The transition state is assumed, and no hypothesis about the protonation of the dianion or any intermediates is made here.

of an A at this site supports self-cleavage, albeit at a rate 2−3 orders of magnitude slower than that of the wild-type ribozymes. Comparison of the kinetic pH profiles of the wild-type ribozymes and the C75A/C76A point mutants showed a difference in the apparent pK_a of the reaction by approximately the same amount as in free C versus A nucleosides (~0.7 pH unit).[5−7] In addition, the activity of C76U and C76 deletion forms of the antigenomic ribozyme can be partially restored by addition of exogenous imidazole or 4-substituted imidazole analogues as well as cytosine and isocytosine. The pH profiles of the 4-substituted imidazole reactions show apparent pK_a's similar to those of the free imidazole analogues. Kinetic solvent isotope effect (KSIE) and proton inventory experiments confirmed that a single proton is transferred during the rate-limiting step of the reaction.[8] These data support a role for C75/C76 as a general acid or general base in the transesterification reaction.

For efficient catalysis, the pK_a of C75 would have to be near 7 at some stage of the reaction, as is the case for histidines 12 and 119 in RNase A,[9] enabling C75 to donate or accept a proton at physiological pH. While the pK_a of free cytidine is 4.2,[7] cytosines with shifted pK_a's have been observed in RNA and DNA.[10−18] In these studies, nuclear magnetic resonance (NMR) was used to detect the protonated N3 imino proton, which is usually stabilized through a hydrogen bond with an acceptor group in a non-Watson−Crick base-pair interaction. Methylation by dimethyl sulfate (DMS) of N3 on cytosine[19] and N1 or N3 on adenosine[20] is also sensitive to protonation, and bases with unusually shifted pK_a's have recently been detected using

nucleotide analogue interference mapping.[21] Using these techniques, bases with unusually shifted pK_a values have been observed adjacent to the active sites of the lead-dependent ribozyme[22] and the hairpin ribozyme,[23] and in the peptidyl transferase center of the ribosome.[20,24]

To measure the pK_a of C75 in the active site of the genomic HDV ribozyme directly, we designed a trans-acting construct analogous to one previously described[25] (Figure 2a). This allowed site-specific $^{13}C,^{15}N$-labeling of individual residues. We show that the in situ pK_a of C75 in the product form of the HDV ribozyme, as calculated from ^{13}C chemical shifts of the base carbons, is only modestly shifted from that of free cytidine. Mechanistic implications of this finding are discussed.

Materials and Methods

Preparation of Site-Specifically $^{13}C,^{15}N$-Labeled HDV Ribozymes. The two RNAs that comprise the NMR construct were transcribed in vitro. The 5′ RNA was transcribed from a *Bsa*I-linearized pUC19-derived plasmid containing the T7 promoter and the ribozyme coding sequence, using a standard protocol for in vitro transcription;[26] the 3′ RNA was transcribed separately from a synthetic DNA duplex in which the last two coding nucleotides were 2′-methoxylated to prevent addition of untemplated nucleotides to the transcript.[27] The 3′ RNA was transcribed with a limiting $^{13}C,^{15}N$-labeled CTP (Martek Biosciences, Cambridge Isotope Laboratories). The initial CTP concentration was 250 μM, while the concentration of ATP, GTP, and UTP was 5 mM each. The incorporation of the labeled CTP during transcription was monitored using anion-exchange chromatography (Dionex NucleoPac PA-100), and the reaction was terminated when no free CTP could be detected. Both RNAs were purified using preparative-scale denaturing polyacrylamide gel electrophoresis. After electroelution and concentration, the RNAs were assembled by heating to 65 °C for 5 min and annealing at 37 °C for 1 h in 10 mM $MgCl_2$, 100 mM LiCl, and 10 mM potassium cacodylate, pH 7. Following self-cleavage, the product complex was purified away from the precursor and the individual RNAs using preparative size-exclusion chromatography (Superdex 75; Pharmacia Biotech, Uppsala, Sweden) in the presence of 100 mM LiCl, 1 mM $MgCl_2$, and 10 mM KH_2PO_4/K_2HPO_4, pH 7. The purity of the complex was confirmed using analytical denaturing gel electrophoresis (Figure 2b). The sample was concentrated and exchanged into the final

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pK$_a$ Measurement in a Genomic HDV Ribozyme

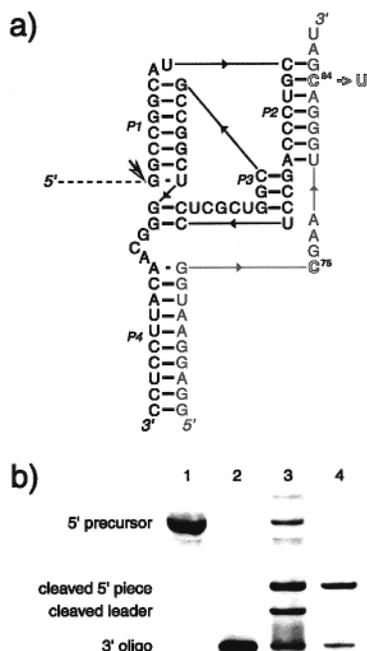*J. Am. Chem. Soc., Vol. 123, No. 35, 2001* 8449

Figure 2. NMR construct and purification. (a) Sequence and secondary structure of the trans construct used in the NMR analysis. The sequence is based on the genomic HDV ribozyme, and the secondary structure reflects the double-pseudoknot observed in the crystal structure of the ribozyme.[4] Dark letters correspond to the nucleotides of the 5′ RNA. The dotted line corresponds to the leader sequence which is cleaved off during the reaction; the arrow indicates the cleavage site. Gray letters correspond to the 3′ RNA with outlined ¹³C,¹⁵N-labeled cytosines and C84U mutation made in the control construct. (b) Analysis of the self-cleaved complex. RNA samples were fractionated on a 16% denaturing polyacrylamide gel. Precursor 5′ RNA (lane 1) and 3′ RNA (lane 2) were heated in the presence of magnesium at 65 °C and anealed at 37 °C to facilitate trans cleavage (lane 3). The product complex was purified using size-exclusion chromatography (lane 4).

NMR solution containing 10% D₂O using Centricon 10 concentrators (Amicon). The final concentration of all NMR samples was between 0.5 and 1.5 mM. The NMR samples were stable for several months and produced almost identical ¹H NMR spectra after heating to 40 °C and lyophilization (data not shown). The imino regions of the ¹H NMR spectra were also virtually identical to spectra obtained from a sample made with a synthetic 3′ oligomer (Dharmacon). All pH values were measured using a glass electrode placed directly in the NMR sample; no correction was made for the 10% D₂O in the samples. To adjust the pH, the samples were titrated with 0.1 M LiOH and 0.1 M H₃PO₄. Titration with KOH and HCl did not result in noticeable changes in the proton spectra, as compared with the LiOH/H₃PO₄ titration, suggesting that the structure of the ribozyme is not dependent on the type of acid or base used in the titration. Three other NMR samples were prepared: (1) a construct in which the 5′ RNA strand started with G(+1) 5′-monophosphate, (2) an inhibited form of the ribozyme with a deoxy(A) at the −1 position, and (3) an uncleaved form of the ribozyme in which the 5′ and 3′ pieces were assembled and purified in the absence of hydrated divalent metal ions. All three samples exhibited ¹³C chemical shifts for the C75 that were similar to those of the cleaved form at neutral pH, but upon acid titration the quality of the ¹³C spectra decreased substantially. In all cases the lines shifted, broadened, and split into multiple components, thereby precluding a straightforward pK$_a$ analysis.

NMR Spectroscopy. The ¹H-decoupled ¹³C spectra were recorded on a Varian Inova 500 spectrometer at 125.885 MHz using a ¹³C direct-detect probe with 2.5 s ¹H NOE presaturation. The spectra were recorded at 37 °C with 7168 complex points with a sweep width of 18 002 Hz and the carrier at 109.3 ppm. Typically, from 500 to several thousand scans were recorded to obtain an adequate signal-to-noise ratio.

Table 1. pK$_a$ Values of ¹³C,¹⁵N-labeled Cytosines under Varying Metal Ion Conditions

position	pK$_a$[a]	solvent conditions
C75	5.4	0.7 mM CaCl₂, 70 mM LiCl
C75	4.8−5.0	∼0.7 mM MgCl₂, 70 mM LiCl
C75	4.5−4.7	0.9 mM MgCl₂, 90 mM LiCl
C75	4.1−4.3	3 mM MgCl₂, 40 mM LiCl
C84	3.5−3.6	∼0.7 mM MgCl₂, 70 mM LiCl

[a] The pK$_a$ values represent averages of the pK$_a$'s calculated from the C2 and C4 carbon chemical shifts. All values for the Mg²⁺ conditions were generated by fitting eq 2 to the data with pK$_a$ as a variable. δ_{max} for the C2 and C4 resonances of CMP is 9.4 and 7.1, respectively.[28] These values are used to calculated the lower limit of the pK$_a$ range. The upper limit of the pK$_a$ range is calculated with 30% lower values of δ_{max}. The calculations for the Ca²⁺ condition included δ_{max} as a variable, giving the values of 8.1 and 5.9 for C2 and C4 resonances, respectively.

Determination of pK$_a$ of C75. The pK$_a$'s were determined from ¹³C chemical shifts using the equation

$$\delta_{observed} = \frac{\delta_{protonated} + \delta_{deprotonated} \times 10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (1)$$

where $\delta_{protonated}$ and $\delta_{deprotonated}$ are the chemical shifts of protonated and deprotonated states, respectively.[23] A more convenient form of the equation for fitting our experimental data is obtained by essentially referencing the chemical shifts of each observed carbon to their high pH value. The equation then becomes

$$\Delta\delta = \delta_{observed} - \delta_{deprotonated} =$$
$$\frac{\delta_{protonated} - \delta_{deprotonated}}{1 + 10^{(pH-pK_a)}} = \frac{\delta_{max}}{1 + 10^{(pH-pK_a)}} \quad (2)$$

where δ_{max} is the full change in chemical shift of the carbon resonance upon protonation. When full protonation of the titrated residue is not observed, both pK$_a$ and δ_{max} have to be estimated from the available data. In most of our titrations, unambiguous ¹³C spectra could only be observed up to about the midpoint of the titration. In the cases where only partial protonation of the base was observed, estimation of both pK$_a$ and δ_{max} resulted in unrealistically high values of δ_{max}. In such cases the pK$_a$ was calculated while assuming the value of δ_{max} to be the same as that in free nucleoside monophosphate, as was observed in a lead-dependent ribozyme.[28] A similar δ_{max} has been reported for cytosine in a CACACA hexanucleotide in D₂O, although an about 20% lower δ_{max} is observed with the same molecule in dimethyl sulfoxide solution.[29] Somewhat lower δ_{max} values have been reported for adenosines with unusually shifted pK$_a$'s in the hairpin ribozyme, as compared with a lead-dependent ribozyme, although full deprotonation was not observed.[23] Lowering δ_{max} increases the estimated pK$_a$ of the titrated base. The effect of decreased δ_{max} on the pK$_a$ of C75 is reflected in Table 1.

Results

Design and Preparation of the Construct. In the two-piece RNA construct used in this study, the 5′ RNA contains a leader sequence followed by the first 52 nucleotides of the ribozyme in which the P4 helix is shortened and the loop L4 left out in order to allow for a break in the backbone (black letters in Figure 2a). The 3′ RNA is a 23-nucleotide oligomer that base-pairs with the 5′ strand in regions P2 and P4 to form an active ribozyme (gray in Figure 2a). The G10-C85 base pair (wild-type genomic ribozyme numbering) is inverted, leaving the 3′ RNA with only two cytosines. This allows for selective incorporation of ¹³C,¹⁵N-labeled cytosines at the active site

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(position 75 in the wild-type genomic ribozyme) and, as a control, in helix P2 (position 84). To assign the carbon resonances of the labeled cytosines, a separate construct was prepared with a C-to-U mutation at position 84 (Figure 2a). The annealed complex cleaved to completion when stoichiometric amounts of the two RNAs were mixed, and the resulting product was purified under native conditions from the precursor and the cleaved leader sequence (Figure 2b). To ensure all of the $^{13}C,^{15}N$-labeled 3′ strand was involved in complex formation, the NMR samples were prepared with excess 5′ strand, and the self-cleaved complex was purified as described above. The samples were prepared in 1 mM MgCl$_2$, 100 mM LiCl, and 10% D$_2$O. The HDV ribozyme requires the presence of divalent metal ions to maintain its tertiary fold;[30–32] lithium was chosen to prevent formation of alternative structures by the G-rich 3′ RNA strand.

NMR Spectra of the Self-Cleaved Form of the Trans HDV Ribozyme. Initial ^1H NMR characterization of the self-cleaved form of the ribozyme revealed a well-dispersed imino region of the spectrum with about 20 distinguishable peaks (Figure 1S, Supporting Information). No proton resonance in the 15 ppm region was observed at any pH or temperature, indicating that solvent exchange with any potential imino protons of unusually basic cytosines was fast on the NMR time scale. However, two amino resonances with a pH- and temperature-dependent chemical shift were observed (p$K_a \sim$7−7.5). Specific isotopic labeling of the C75 confirmed that neither of these ^1H resonances belongs to the amino group of C75. In the absence of full assignment of the molecule, we hypothesize that the observed pH- and temperature-dependent spectral change resulted from a structural rearrangement, most likely in the base quadruple involving protonated C41.[33,34]

We were able to determine the pK_a of C75 using indirect detection of the base protonation through base carbon chemical shift measurements. The downfield ^{13}C spectral region of the constructs used in this study is presented in Figure 3. This region contains resonances corresponding to carbons 2, 4, and 6 on the cytosine ring. Positions 2 and 4 are not protonated and exhibit the highest chemical shift change upon protonation of the N3 imino nitrogen.[28,29] Figure 3a shows the ^{13}C spectra of cytosines 75 and 84 at acidic to neutral pH's. The spectra contain two peaks for each carbon type, one of which shows a more dramatic chemical shift change as a function of pH (indicated by the solid line in Figure 3a). Comparison with the spectrum of the C84U mutant (Figure 3b) demonstrates that these shifting peaks correspond to the C75 carbon resonances. The resonances of C84 (indicated by the dotted line in Figure 3a) exhibit only a slight change of chemical shift. This nucleotide is involved in a Watson−Crick base-pair interaction,[4] which stabilizes the deprotonated state of the N3 of cytosine.

Measurement of the pK_a of the Active-Site Cytosine. Figure 4 shows the titration curves of the C2 carbons on the $^{13}C,^{15}N$-labeled cytosines under various salt conditions. The pK_a values were calculated by fitting the chemical shift data to eq 2, with pK_a as a variable. In only one case, in the presence of calcium, could the sigmoidal shape of the titration curve be observed. In that case, both δ_{max} and pK_a were used as variables to fit the

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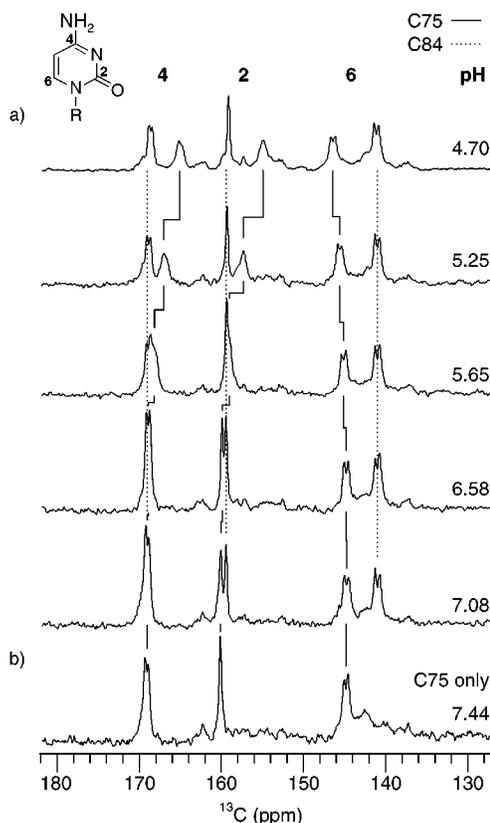

Figure 3. Carbon-13 NMR spectra of the selectively labeled HDV ribozymes. (a) C75- and C84-labeled complex at pH 4.70, 5.25, 5.65, 6.58, and 7.08. (b) Complex with C84U mutation showing a single ^{13}C resonance for each carbon type at pH 7.44. Smaller peaks correspond to the natural abundance ^{13}C spectrum of the 74 unlabeled nucleotides. Dotted lines and solid lines connect resonances of carbons at positions 2, 4, and 6 of C84 and C75, respectively. All spectra were collected at 37 °C and processed with 20 Hz line broadening.
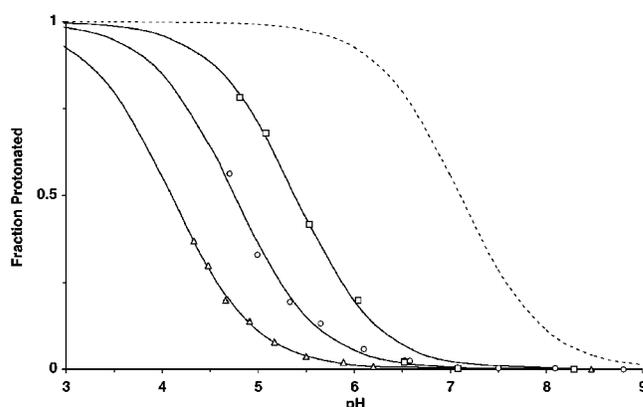

Figure 4. Protonation of C75. The chemical shifts of the C2 carbons were referenced to the highest pH measured and to the maximum chemical shift, δ_{max}, used to fit the data (see Table 1). Sample conditions were as follows: 0.7 mM MgCl$_2$, 70 mM LiCl (circles); 3 mM MgCl$_2$, 40 mM LiCl (triangles); 0.7 mM CaCl$_2$, 70 mM LiCl (squares). Lines represent the best-fit curves generated using eq 2. Dashed line is a theoretical curve based on kinetic data representing the protonation of C75 in the wild-type genomic ribozyme in 0.87 mM MgCl$_2$ at 37 °C.[6]

data, resulting in a δ_{max} which was about 15% lower than the δ_{max} of CMP.[28] As a result of this change, we used the range of $0.7−1.0 \times \delta_{max}$ (CMP) to determine the range of values for pK_a's in the presence of magnesium. The calculated pK_a's are shown in Table 1. The values represent the average of pK_a's calculated from chemical shifts of the C2 and C4 carbons. The

difference in pK_a values calculated with C2 versus C4 chemical shifts is 0.04−0.06.

Discussion and Conclusions

In this study, we used a ^{13}C-labeled form of the genomic HDV ribozyme to determine the pK_a of an active-site cytosine proposed to be involved in general acid/base catalysis during ribozyme self-cleavage.[4−6,8] In order for C75 to be an efficient general acid/base catalyst in the phosphodiester cleavage reaction, its pK_a should be near 7 at some stage during ribozyme self-cleavage to enable it to accept or donate a proton.

^{13}C NMR is perhaps the most sensitive method to measure the protonation state of specific functional groups in macromolecules. While ^{1}H NMR can be used to detect a titrating proton directly, usually in combination with indirect ^{15}N chemical shift observation, this is only possible under a slow-exchange regime and in cases where the ^{15}N relaxation is slow enough to allow for efficient magnetization transfer. Direct detection of the heteronuclei on the titratable nucleobase circumvents the problem of rapid relaxation in large molecules and rapid solvent exchange. For this purpose, ^{13}C detection is superior to ^{15}N observation, given the higher receptivity of ^{13}C and the large chemical shift range for the base carbons that are directly bonded to the titrated imino groups.[28,29] The sensitivity of these ^{13}C chemical shifts to pH makes this method an especially informative probe of the state of protonation of the site of interest.

^{13}C spectra of the site-specifically labeled HDV RNA show that, in the product state of the reaction, the pK_a of C75 is only moderately shifted from that of the free nucleoside. The pK_a estimated from the ^{13}C chemical shifts of the C2 and C4 carbons of the C75 base is about 4.8 when measured in 0.7 mM Mg^{2+} and 70 mM Li^{+}, 0.6 unit above the pK_a of free cytidine. The pK_a of the same group measured at 3 mM Mg^{2+} is not shifted from that of free nucleoside at all, while in the presence of 0.7 mM Ca^{2+} it is slightly higher (5.4). As a control experiment, titration of 1 mM cytidine nucleoside was carried out in samples having between 0 and 3 mM MgCl$_2$ in the background of 70 mM LiCl. Using the chemical shifts of the H5 and H6 protons, the pK_a of the base was found to be independent of the divalent metal ion concentration under these conditions. Our results show that, in the product state of the ribozyme, C75 is fully deprotonated close to neutral pH. The general trend of the microscopic pK_a shifts of C75 follows the same trend in apparent reaction pK_a's:[6] the pK_a shifts downward with increasing concentration of Mg^{2+} and upward in the presence of Ca^{2+} (Table 1). However, the microscopic pK_a's measured in the self-cleaved form of our construct are about 2 units lower than the apparent pK_a's calculated from the kinetic pH profiles of the cis-acting wild-type genomic ribozyme under similar conditions (Figure 4).[6]

Our data indicate that if C75 is involved in general acid/base catalysis, its pK_a must be higher in the reactant or intermediate state of the cleavage reaction. We have attempted to measure the pK_a of the C75 in the uncleaved form of the ribozyme. While we were able to purify the assembled trans complex in the presence of EDTA and cobalt hexammine (an inhibitor of the cleavage reaction), the lack of well-dispersed ^{1}H spectra indicated that the complex was not in a unique conformation and, close to neutral pH, the C2 and C4 resonances of the specifically labeled C75 had chemical shifts similar to those of the deprotonated cytosine in the self-cleaved ribozyme (Figure 3S, Supporting Information). The lack of unique resonances corresponding to the base carbons precluded a pK_a

analysis of this form of the ribozyme, although we estimate that the pK_a is less than 5 (in 1 mM EDTA, 1.5 mM cobalt hexammine, and 150 mM LiCl). A related construct of the ribozyme, which contained a 5′ monophosphate at the cleavage site, exhibited a well-dispersed ^{1}H spectrum similar to the ^{1}H spectrum of the cleaved form of the ribozyme. The ^{13}C spectra, on the other hand, showed multiple peaks for each type of carbon on the cytosine ring, barring pK_a analysis in this construct as well. As in the case of the uncleaved form of the ribozyme, near neutral pH the ^{13}C chemical shifts were similar to those of a deprotonated cytidine (in 0.9 mM MgCl$_2$ and 90 mM LiCl). This suggests that the C75 may not have an unusually shifted pK_a in the precursor state of the ribozyme.

To understand these results in light of the kinetic data published by other groups requires consideration of the general mechanism of RNA cleavage (Figure 1). Breaker and co-workers have investigated the influence of the 2′ nucleophile on the rate of phosphodiester cleavage and have concluded that the maximum rate of cleavage with the fully deprotonated 2′ hydroxyl group is about 0.01 min^{-1}.[35] In addition, when the conformation of the 2′ hydroxyl and the reactive phosphate group is close to the theoretical geometry of an in-line attack, the rate of cleavage is accelerated by about 10-fold.[36] The observed maximum rate of cleavage of the HDV ribozymes is about 10 min^{-1}, about 100-fold higher than the maximum theoretical rate resulting from the nucleophilic attack by the 2′ oxyanion group. This means that further acceleration of transesterification must come from stabilization of the transition state and/or stabilization of the leaving group. The general acid/base mechanisms proposed previously have suggested stabilization by protonation of the 5′ oxyanion leaving group as one of the steps involved in the catalysis,[6,8] although protonation of an anionic phosphorane intermediate related to the transition state cannot be ruled out.

There are three basic possibilities that would explain the equilibrium data presented in this paper in view of the available kinetic data for the HDV ribozymes: (1) if the observed reaction pK_a's reflect a rate-limiting step other than chemistry, C75/C76 may not be a general acid or base in the transesterification reaction; (2) if none of our precursor and precursor analogue samples had properly preorganized active sites, the pK_a of C75 immediately before the reaction may in fact be significantly shifted, reflecting the apparent reaction pK_a; (3) if our precursor NMR samples had active sites resembling the correct precleaved state, the apparent reaction pK_a may reflect transient protonation of C75 during an intermediate step of the reaction. We now consider the evidence for or against each of these scenarios.

The apparent reaction pK_a's reflect the pK_a of an active-site group only if the rate-limiting step of the reaction represents a chemical step in the transesterification. The wealth of HDV ribozyme kinetic data indicates that during the observed step of catalysis, there is at least one, and in most cases only one, proton transferred, in contrast to the six proton transfers hypothesized for an analogous reaction catalyzed by RNase A.[37] Proton inventory and kinetic solvent isotope effects on the cleavage reaction support a single-proton transfer in the rate-limiting step of the reaction.[8] The ΔpK_a for the self-cleavage reaction in D$_2$O versus H$_2$O was about 0.4 and 0.7 for the genomic and anti-genomic ribozymes, respectively,[6,8] while the ΔpK_a of the free cytidine is 0.53 (Figure 2S, Supporting Information). While these effects demonstrate a general proton

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transfer, they could reflect a structural rearrangement involving formation or breakage of a hydrogen bond during the reaction, rather than reaction chemistry. However, imidazole rescue experiments performed with a variant of the antigenomic ribozyme lacking C76 retained the kinetic solvent isotope effect observed with the wild-type ribozyme, making a structural rearrangement involving C76 unlikely. Furthermore, the apparent pK_a's for the reaction have been shown to be the same for trans cleavage of either a 3′–5′ or 2′–5′ phosphodiester in the anti-genomic ribozyme, even though the observed rates of cleavage were substantially different.[38] These pK_a's are near the pK_a's measured in the wild-type cis ribozymes. These results, and the results obtained with the C75A/C76A mutants described above, strongly suggest that C75/C76 is directly involved in a rate-limiting chemical step in the transesterification, as opposed to a folding transition.

Since our ^{13}C NMR measurements demonstrate that the pK_a of C75 is only moderately shifted in the product state, the apparent reaction pK_a must correspond to a microscopic pK_a either in the precursor active site or in a transition-state-related intermediate. In the reactant state, we detected no protonation of C75 near neutral pH in three different constructs with active sites containing a 5′ phosphate, a 5′ phosphate with an upstream deoxynucleotide, or a 5′ phosphate with an upstream ribonucleotide (inhibited from self-cleaving by cobalt hexammine). The lack of protonation might result from these constructs being improperly folded. However, this is unlikely, since they were assembled under conditions similar to those in which the all

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RNA construct assembles and is catalytically active; and the imino regions of the proton spectra of the 5′phosphate- and 5′deoxyribonucleotide-containing constructs were well dispersed throughout the pH titration. Thus, it appears that the pK_a of C75 in the reactant state is not significantly shifted. Therefore, the unusual shift in the pK_a of C75 obtained from kinetic pH profiles of the transesterification reaction likely demonstrates a rate-limiting, short-lived pK_a shift more closely associated with the phosphorane intermediate, related to the trigonal bipyramidal transition state of the reaction (Figure 1).

Supporting Information Available: Fiure 1S, imino region of the ^{1}H spectrum of the self-cleaved ribozyme; Figure 2S, deuterium isotope effect on pK_a of cytidine; Figure 3S, downfield region of the ^{13}C spectrum of the uncleaved form of the ribozyme at pH 6.2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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