Proton Inventory of the Genomic HDV Ribozyme in Mg^{2+}-Containing Solutions

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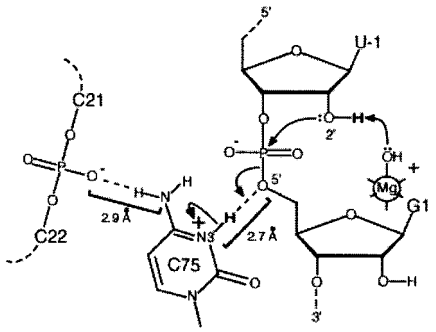
Received July 23, 2001

The mechanism of phosphodiester bond cleavage has been investigated in-depth for a variety of nonenzymatic and enzymatic systems, including protein enzymes and enzyme mimics.[1] Metal ion coordination and proton transfer have been demonstrated as key strategies to accelerate catalysis. Proton inventory studies revealed two proton transfers for the second step of catalysis by ribonuclease A, which involves hydrolysis of a 2′,3′-phosphate ester to a 3′-phosphate.[2] Also, a two-proton inventory was found for hydrolysis of a cyclic phosphate to a phosphate monoester by a cyclodextrin bis(imidazole) enzyme mimic.[3]

The mechanisms of phosphodiester bond cleavage by catalytic RNAs, or ribozymes, have also been investigated.[4] The large (≈400 nucleotides) self-splicing group I intron from *Tetrahymena* uses three Mg^{2+} ions to facilitate bond cleavage, and catalytic roles for Mg^{2+} ions have been found for self-splicing group II introns.[5] In these cases metal ions act primarily as Lewis acids to stabilize developing charge. In smaller ribozymes (≈30−80 nucleotides), the catalytic contribution of metal ions is less certain. Studies have revealed that the hammerhead, hairpin, VS1, and hepatitis delta virus (HDV) ribozymes can cleave reasonably well without divalent ions.[6−8] Recent studies do, however, implicate proton transfer as important in catalysis by the HDV ribozyme.[7,9]

The HDV ribozyme is an 85 nucleotide self-cleaving RNA found in closely related genomic and antigenomic forms.[10] The ribozyme cleaves the phosphodiester bond between U-1 and G1, and results in 5′-OH and 2′,3′-cyclic phosphate termini. The crystal structure of the self-cleaved genomic ribozyme was solved at 2.3 Å resolution and revealed that O5′ of G1 and N3 of C75 are only 2.7 Å apart.[11] This suggested that C75 may function as the general acid in bond cleavage (Scheme 1), and this model was supported by kinetic and mutagenic data.[7,12] A kinetic solvent isotope effect implicated proton transfer in the mechanism,[7] as did rescue of inactive C76/C75 mutants by imidazole.[7,9] In addition, studies of the Mg^{2+} dependence of self-cleavage revealed a role for hydrated magnesium hydroxide as the general base (Scheme 1).[7,8] In this paper we test the mechanism for phosphodiester bond cleavage by the HDV ribozyme further, and examine the number of proton transfers in the transition state for the rate-limiting step.

Scheme 1. Proposed Proton Transfers in Bond Cleavage[a]



[a] A concerted transition state for a one-step mechanism is shown here for simplicity. The actual mechanism, however, may involve a series of steps, some of which may be concerted.

A useful method for determining the number of proton transfers is the proton inventory technique.[13] This method relies on measuring the rate in H_2O/D_2O mixtures and examining the resulting curves. This can help resolve whether one or two proton transfers occur in the rate-limiting step. We carried out these experiments at pL 8, which is in the plateau region of the pL−rate profile (Figure 1).[14] At both 0.87 and 10 mM Mg^{2+}, substantial solvent isotope effects (4.3- to 3.6-fold) were observed, as were pK_a shifts of ≈0.3 units (Figure 1). These pK_a shifts are similar to those expected from ammonium groups.[13] The observed pK_a values in H_2O and D_2O were approximately one unit higher in 0.87 than 10 mM Mg^{2+}. This appears attributable to Coulombic repulsion between protonated C75 and Mg^{2+} in the reactant state.[7] These results suggest that chemistry is rate limiting, and the sizable isotope effects afforded an experimentally tractable inventory.

Proton inventories at 0.87 and 10 mM Mg^{2+} are shown in Figure 2. In both cases, plots of relative rate constants versus D_2O fraction (n) were concave, or "bowl-shaped". The data were fit to the Gross−Butler equation (eq 1) for a two-proton inventory,

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(4) For reviews see: (a) Narlikar, G. J.; Herschlag, D. *Annu. Rev. Biochem.* **1997**, *66*, 19−59. (b) Cech, T. R.; Golden, B. L. In *The RNA World*, 2nd ed.; Gesteland, R. F., Cech, T. R., Atkins, J. F., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1999; pp 321−349. (c) Takagi, Y.; Warashina, M.; Stec, W. J.; Yoshinari, K.; Taira, K. *Nucleic Acids Res* **2001**, *29*, 1815−1834.

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(12) Another possible mechanism implicates C76 (the antigenomic counterpart of C75) in proton transfer, but as the general *base* in the reaction.[9] This mechanism is kinetically equivalent to Scheme 1, and has not been formally ruled out. However, it does appear less likely based on the crystal structure, results on the Mg^{2+}-free reaction, and anticooperative interactions between Mg^{2+} and H^+ binding to the precursor ribozyme.[7] Importantly, if this alternate mechanism is correct, the result herein of two proton transfers still holds. Similarity in secondary structures[10] and mechanistic features[7,9] does suggest that the genomic and antigenomic ribozymes employ highly similar mechanisms.

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(14) The plateau region is typically used for the proton inventory wherein the general acid and base are thought to be in or near their fully functional forms.[2,3] This approach avoids the complication of having to correct the data for pK_a differences in the two solvents. (See: Sawata, S.; Komiyama, M.; Taira, K. *J. Am. Chem. Soc.* **1995**, *117*, 2357−2358.) For the mechanism favored here, the plateau region is due to compensation of the loss of the functional protonated form of C75 by gain of the functional deprotonated form of hydrated Mg^{2+}. Nevertheless, effects of D_2O on pK_as and Brønsted acidity and basicity of the general acid and base are likely to be very similar and cancel. pL values higher than 8 were not used since these can cause deprotonation of unperturbed RNA functional groups and lead to cleavage of all phosphodiester bonds.

10.1021/ja0166850 CCC: $20.00
Published on Web 10/23/2001

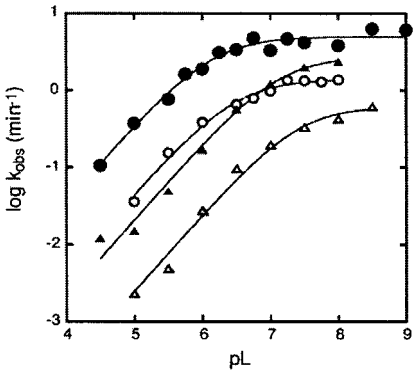

Figure 1. Reactivity−pL (−pH or −pD) profiles for 0.87 mM Mg^{2+} in H_2O (▲) ($pK_a = 7.12 \pm 0.15$), 0.87 mM Mg^{2+} in D_2O (△) ($pK_a = 7.40 \pm 0.11$), 10 mM Mg^{2+} in H_2O (●) ($pK_a = 6.15 \pm 0.06$), and 10 mM Mg^{2+} in D_2O (○) ($pK_a = 6.49 \pm 0.05$). Data were fit as described[7] by using the log of the equation $k_{obs} = k_{max}/[1 + 10^{(pK_a - pH)}]$. All experiments were conducted at 37 °C, as described.[7,8] D_2O-containing solutions were prepared as described.[13]

assuming equivalent transition state fractionation factors (ϕ^T) for both transfers.[13,15] The modest downward curvature of the plot is

$$k_n/k_0 = (1 - n + n\phi^T)^2 \qquad (1)$$

typical of other two-proton inventories.[2,3] To test the fit further, we plotted the square root of the relative rate constants versus D_2O fraction. In both cases, the data were well fit by a straight line going through $y = 1$. In the case of a one-proton inventory, the square-root plot should be convex,[3] which is clearly not observed. Overall, graphical analyses are inconsistent with a one-proton inventory, and consistent with a minimum two-proton inventory. A two-proton inventory is consistent with the transition state in Scheme 1, although a more complex sequential mechanism with a phosphorane intermediate[16] has not been ruled out. Requirement of two proton transfers for phosphodiester bond cleavage may be seen as a selective advantage to the stability of the phosphodiester bond since spontaneous cleavage is less likely.

Both fractionation factors are within the range expected for proton transfer among O and N.[17] A larger fractionation factor, $\phi^T = 0.609 \pm 0.002$, was observed in 10 mM Mg^{2+}, and a smaller fractionation factor, $\phi^T = 0.481 \pm 0.007$, was observed in 0.87 mM Mg^{2+}. The larger fractionation factor represents a smaller observed isotope effect and is associated with a shallower bowl plot; this may occur because the lower pK_a of C75 in saturating Mg^{2+} (Figure 1)[18] may lead to only partial protonation occurring in concert with deprotonation of the 2′-OH. In contrast, the pK_a of C75 is higher in sub-saturating Mg^{2+}, which may lead to concerted protonation/deprotonation events.

A proton inventory experiment on the antigenomic HDV ribozyme was recently reported by Shih and Been, and resulted in a one-proton inventory.[19] Since it is possible that the cleavage reaction involves a series of sequential steps,[16] their result[19] may reflect a different rate-limiting step of the same mechanism. Differences between our experimental conditions and theirs include genomic versus antigenomic ribozymes, temperatures of 37 versus 25 °C, and absence versus presence of 1 mM spermidine, respectively. In addition, their experiments were at 10 mM Mg^{2+}, and our experiments give a "shallow bowl" under these conditions that is not dissimilar from a straight line (Figure 2A). To our knowledge, this report represents the first demonstration of a two-proton inventory for cleavage of a 3′,5′-phosphodiester bond.

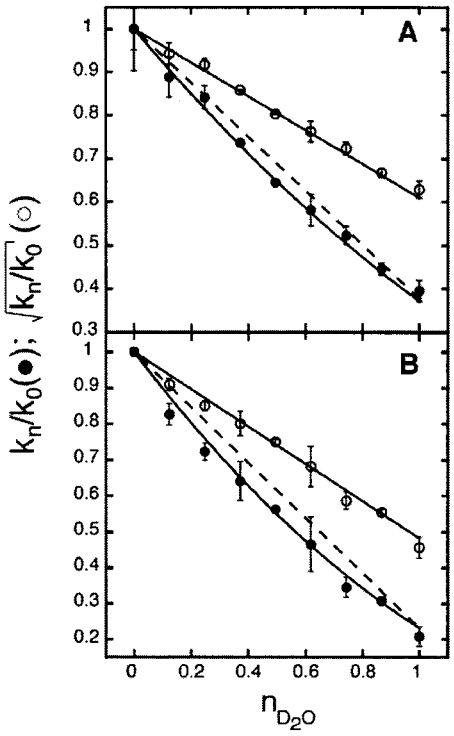

Figure 2. Proton inventories for (A) 10 mM Mg^{2+} at pL 8.0 (●) and (B) 0.87 mM Mg^{2+} at pL 8.0 (●). Each point is the average of 2−5 independent trials. The *y*-axis is the ratio of the observed rate constant in an H_2O/D_2O mixture (k_n) to that in 100% H_2O (k_0); the *x*-axis is the atom fraction of D_2O (n_{D_2O}). The difference in the density of H_2O and D_2O was taken into account.[13] Data were fit to eq 1 for a two-proton inventory, assuming equal transition state contributions for the two transfers. The isotope effect k_H/k_D for each of the proton transfers was 1.64 (A) and 2.08 (B). These values are similar to those found for hydrolysis of 2′,3′-cyclic phosphates.[2,3] Also shown are secondary plots of the square root of k_n/k_0 vs n_{D_2O}, (○). These data were fit to a straight line with a *y*-intercept of unity. Data fitting was by nonlinear least squares, using standard deviations as weights. In both panels, dashed lines connecting k_n/k_0 values in 100% H_2O and 100% D_2O are shown for comparison to theoretical one-proton inventories.

Proton transfer by the HDV ribozyme involves one transfer from a side chain (cytosine) and one to an exogenous metal ion. In this sense, the HDV ribozyme is similar to one-half of RNase A, which uses *two* protein side chains (histidines) with pK_as of neutrality in proton transfer.[1b] RNA may be viewed as an adept catalyst with the ability to provide a microenvironment that shifts the pK_a of a nucleobase to neutrality to act as a general acid, and/or to recruit a metal ion to serve as the general base. The ability of ribozymes to employ multiple proton transfers in catalysis suggests that there may be a diverse set of reactions RNA once catalyzed or can be made to catalyze.

Acknowledgment. We thank members of the Bevilacqua lab and Professor T. Glass for comments on the manuscript. This research was supported by a Fellowship from the Alfred P. Sloan Foundation and a Camille Dreyfus Teacher−Scholar Award to P.C.B.JA0166850

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(15) Hydrogen bonding groups typically have reactant state fractionation factors ≈1, and so were not included in eq 1.[13] Transition state protons contribute the inverse of their fractionation factors to the solvent isotope effect. Fits to a kinetic model involving non-equivalent isotope effects for the two transfers were not statistically better compared to a model with equivalent effects, and so were not used.

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