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# Detection of a Proton-Transfer Process by Kinetic Solvent Isotope Effects in $NH_4^+$-Mediated Reactions Catalyzed by a Hammerhead Ribozyme

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Hammerhead (HH) ribozymes are among the natural catalytic RNAs, whose sequence motif was first recognized in the satellite RNAs of certain viruses.[1] Engineered *trans*-acting HH ribozymes, consisting of antisense sections (stems I and III) and a catalytic core with a flanking stem-loop II section, have been used in mechanistic studies.[2] HH ribozymes cleave their target RNAs at specific sites, as shown in the upper left corner of Figure 1, via a pathway in which the 2′-OH attacks the adjacent scissile phosphate, acting as an internal nucleophile [via putative transition state 1 (TS1)], and, then (or in concert with the attack by the 2′-OH), the 5′-oxygen of the leaving nucleotide is released to generate a 3′-end 2′,3′-cyclic phosphate and a 5′-OH terminus [via transition state 2 (TS2)]. This reaction proceeds with inversion of the configuration at the phosphorus atom, suggesting a direct in-line attack with development of a trigonal bipyramidal transition state or intermediate.[3] In nature, HH ribozyme-catalyzed reactions involve $Mg^{2+}$ ions and, thus, HH ribozymes are generally considered to be metalloenzymes.[4] Two mechanisms have been proposed for the chemical cleavage reactions: a single-metal-ion mechanism[5] and a double-metal-ion mechanism.[6] In both mechanisms, it is assumed that the first metal ion assists in removal of a proton from the attacking nucleophile 2′-OH. In the double-metal-ion mechanism, a second $Mg^{2+}$ ion is proposed to act as a Lewis acid catalyst to neutralize the developing negative charge on the leaving 5′-oxygen.[4e,6] However, it was reported recently that HH ribozyme reactions can proceed in the presence only of high concentrations of nonmetal monovalent ions, such as ammonium ions, with rates similar to those of $Mg^{2+}$-catalyzed reactions.[7a] In addition, it is proposed that these cations are not directly involved in the chemical cleavage reaction.[7b,c] Thus, we must ask whether $Mg^{2+}$ ions really act as catalysts in $Mg^{2+}$-mediated HH ribozyme reactions. The possibility of a nucleobase acting as a catalyst has also been discussed on the basis of an analysis of the crystal structure of the complex between an HH ribozyme and its product.[8]

We decided to compare the mechanisms of metal ($Mg^{2+}$ or $Li^+$)-mediated and ammonia-mediated reactions by examining their kinetics. In many RNA-cleavage reactions, not only those catalyzed by proteinaceous enzymes but also those catalyzed by various ribozymes, such as group I and group II introns and genomic HDV ribozymes, the developing negative charge on the leaving group is neutralized either by a proton or by a Lewis acid.[6] In the double-metal-ion mechanism of HH ribozyme-catalyzed reactions, a $Mg^{2+}$ ion coordinates directly with the 5′-oxygen, acting as a Lewis acid catalyst, as shown in the bottom right corner of Figure 1.[4e,6,9] In accordance with this mechanism, no proton-transfer process was detected during measurements of kinetic solvent−isotope effects in $D_2O$ with a 32-mer HH ribozyme (R32) and its 11-mer substrate (S11), which are shown in Figure 1.[9] In $NH_4^+$-mediated reactions, it is possible that an ammonium ion might donate a proton to the leaving 5′-oxygen, acting as a general acid catalyst. Then, in the latter case, it should be possible to detect the proton-transfer process by measurements of kinetic solvent−isotope effects of $D_2O$ similar to those that we made for the analysis of $Mg^{2+}$-mediated HH ribozyme reactions, using the same combination of R32 and S11.[9]

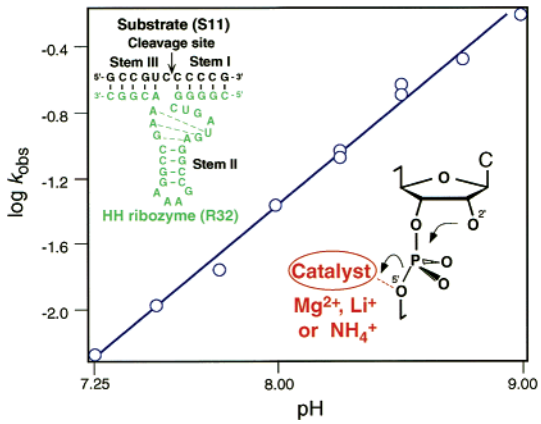


***Figure 1.*** Dependence on pH of the cleavage rate of the hammerhead ribozyme-catalyzed reaction in 4 M $NH_4Cl$. The $NH_4Cl$-containing buffer used in this experiment was Bis-Tris propane and the pH was adjusted to 7.25, 7.50, 7.76, 7.99, 8.25, 8.75, and 8.99. Cleavage rates increased linearly with pH, yielding a slope of 1.22. Upper left: Secondary structure of the complex between the hammerhead ribozyme (green; R32) and the substrate (S11) that were used in this experiment. The arrow indicates the cleavage site. Bottom right: Possible catalytic role of a catalyst. The $Mg^{2+}$ or $Li^+$ ion acts as a Lewis acid catalyst by directly coordinating to the leaving 5′-oxygen. By contrast an ammonium ion acts as a general acid catalyst by donating a proton to the leaving 5′-oxygen. The catalyst acting to remove a proton from the attacking nucleophile, 2′-OH, is omitted.

Before measurements, we had to check the reaction conditions to exclude experimental artifacts as far as possible.[10] First, to determine whether the reaction in the presence of ammonium ions proceeded without contamination by residual amounts of divalent metal ions, we examined the effects of EDTA on cleavage profiles and rates in 50 mM Tris, 2 M $NH_4Cl$, pH 8.00, at 25 °C in the presence and in the absence of 25 mM EDTA. Since the presence of EDTA had no effect on overall cleavage profiles and rates,[11] it seemed very likely that the ribozyme reaction actually proceeded in the presence exclusively of ammonium ions. Next, we checked the pH−rate profile for the ribozyme-saturated single-turnover reaction in 50 mM Bis-Tris propane and 4 M $NH_4Cl$ at 25 °C at from pH 7.25 to 8.99. As shown in Figure 1, the reaction depended

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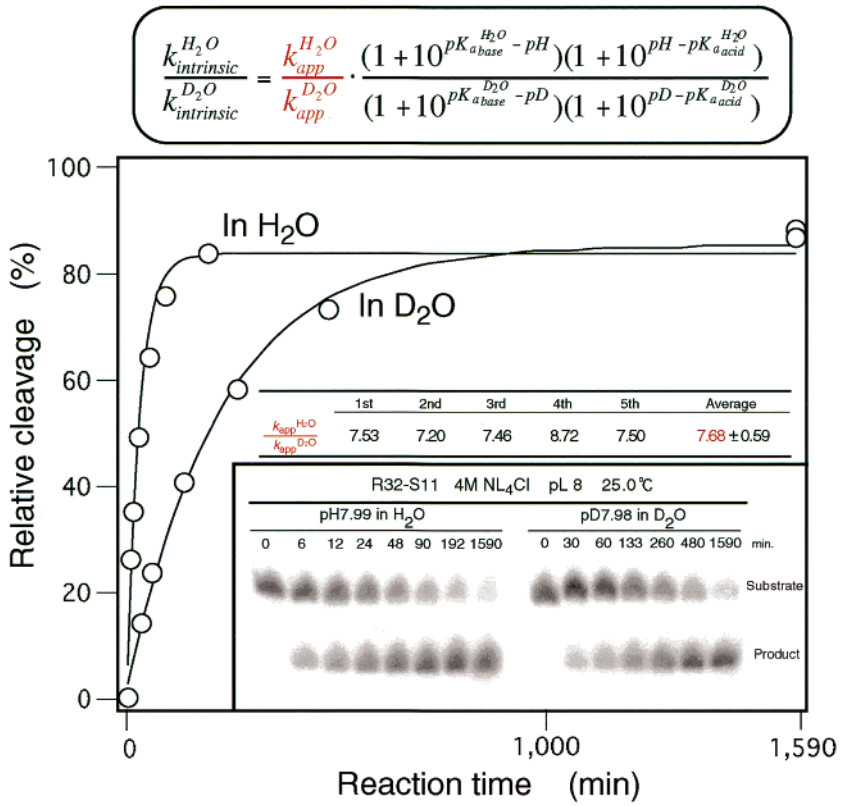


***Figure 2.*** Representative time courses of formation of products. Single-turnover reactions were followed in a reaction mixture that contained 240 nM HH (R32), a trace amount of 5′-$^{32}$P end-labeled substrate (S11), and 4 M $NL_4Cl$ in 50 mM Bis-Tris propane buffer at 25 °C and pH 7.99 or pD 7.98, in $H_2O$ or pure $D_2O$. The observed rates were 0.0335 $min^{-1}$ in $H_2O$ and 0.00445 $min^{-1}$ in $D_2O$. The value for the apparent solvent-isotope effect, $k_{app}^{H_2O}/k_{app}^{D_2O}$, is ~7.53. The least-squares fit to the data was achieved by using a nonlinear curve-fitting program. The apparent solvent-isotope effects are summarized in the inserted table. The equation for conversion of the apparent isotope effect (in red) to the intrinsic one is shown above the profile.

on pH. The slope of the graph in Figure 1 is close to unity, namely 1.22, indicating that the observed rates reflected the rate-limiting chemical step. A similar slope of unity was also obtained in the analysis of $Mg^{2+}$-mediated HH ribozyme reactions,[5b,6e,f,12] reflecting the higher amounts of the active species (deprotonated 2′-oxyanion nucleophile) at higher pH. However, the slope of unity also warns us that care is necessary in the analysis of kinetic solvent−isotope effects at a fixed pL (L = H or D, for the same concentration of protons or deuterium ions) since the fractions of active nucleophiles and catalysts in $H_2O$ and in $D_2O$ are different.[6e,f,9]

We chose the following conditions for examination of the apparent kinetic solvent isotope effect, $k_{app}^{H_2O}/k_{app}^{D_2O}$: 4 M $NL_4Cl$, pL 8 (within the range where the slope was unity), ribozyme-saturated single-turnover conditions, and 50 mM Bis-Tris propane at 25 °C.[13] Representative experimental results are shown in Figure 2, together with the results that yielded an averaged apparent solvent−isotope effect for deuterium of 7.68 ± 0.59 (average from five sets of results). This value (7.68) is significantly larger than the previously determined value of 4.4 for $Mg^{2+}$-mediated reactions with the same ribozyme and substrate.[9] If a nucleobase alone, rather than a $Mg^{2+}$ ion, had acted as a catalyst in both the $Mg^{2+}$-mediated and the $NH_4^+$-mediated reactions, we would expect a similar value for the apparent solvent−isotope effect of deuterium in both reactions. Since the value (7.68) for the $NH_4^+$-mediated reaction was significantly larger than that (4.4) for the $Mg^{2+}$-mediated reaction, it seems more realistic to assume that more protons are transferred in the transition state in the $NH_4^+$-mediated reaction, and that the catalysts might be different in the two reactions. Thus, we should compare the intrinsic isotope effect of the $Mg^{2+}$-mediated reaction with that of the $NH_4^+$-mediated reaction, which can be calculated from the apparent solvent−isotope effects and consideration of the values of $\Delta pK_a$ of the catalyst in $H_2O$ and in $D_2O$, as has been described previously.[6e,f,9,14]

In the simplest model, an apparent solvent−isotope effect is due to two factors: the difference between fractions of catalysts at a fixed pL and the intrinsic solvent−isotope effect. The intrinsic solvent−isotope effect reflects the difference between the energy for transfer of a proton(s) and the energy for transfer of a deuterium ion(s) in the transition state. If the acid and base catalysts in the $NH_4^+$-mediated reaction were $NL_4^+$ and $NL_3$, respectively, the intrinsic solvent−isotope effect, based on the values of $pK_a$ in $H_2O$ and in $D_2O$ of 9.26 and 9.87,[15] can be calculated to be 2.04 from the apparent solvent−isotope effect of 7.68.[16] This intrinsic value is twice the previously calculated value of unity for the $Mg^{2+}$-mediated reaction[6e,f,9] and also for the $Li^+$-mediated reaction (the intrinsic value from three sets of experiments carried out in this study was 1.13). The comparison suggests that the transfer of one proton (or more than one proton) occurs in the transition state of the $NH_4^+$-mediated ribozyme reaction while no proton transfer occurs in the transition state of the $Mg^{2+}$-mediated and $Li^+$-mediated ribozyme reactions. Furthermore, since TS2 is considered to be the overall rate-limiting step in both nonenzymatic and HH ribozyme-catalyzed reactions,[4e,6f,17] the proton-transfer step should be involved with the departure of the 5′-oxygen from the cleavable P−O(5′) bond. In short, it is likely that protonated ammonia works as a general acid catalyst in $NH_4^+$-mediated HH ribozyme-catalyzed reactions.

Taken together, results can most simply be explained as follows (althought we do not completely exclude several other potential interpretations). In the case of the $Mg^{2+}$- or $Li^+$-mediated HH

ribozyme-catalyzed reactions, a $Mg^{2+}$ or $Li^{+}$ ion, respectively, neutralizes the developing negative charge in the transition state by coordinating directly with the leaving 5′-oxygen (see Figure 1). Because of this direct coordination of a $Mg^{2+}$ ion and because of the absence of the transfer of a proton to the leaving 5′-oxygen, the intrinsic solvent−isotope effect is unity in both $Mg^{2+}$- and $Li^{+}$-mediated ribozyme reactions. By contrast, an ammonium ion neutralizes the developing negative charge in the transition state by transferring a proton(s) to the leaving 5′-oxygen (see Figure 1), with the result that the intrinsic isotope effect is 2.04.

In conclusion, our observation of a kinetic intrinsic isotope effect in the $NH_4^{+}$-mediated HH ribozyme-catalyzed reaction supports the hypothesis that (i) transfer of a proton(s) occurs in the transition state, (ii) the catalyst that stabilizes the 5′-leaving group is different from that in the $Mg^{2+}$- or $Li^{+}$-mediated HH ribozyme reaction, insofar as the $NH_4^{+}$-mediated reaction involves transfer of a proton in the transition state while the metal-mediated reaction does not, (iii) an $NH_4^{+}$ ion seems to act as a general acid catalyst in the overall transition state, TS2, and (iv) a nucleobase alone, acting as the catalyst, cannot explain the difference in terms of the solvent−isotope effect between the metal-mediated and $NH_4^{+}$-mediated HH ribozyme reactions. The simplest model that explains the solvent isotope effect is shown at the bottom right in Figure 1.

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(13) The 5 M $NL_4^{+}$-containing buffer, the 5′-end-labeled substrate, and stock solutions of hammerhead ribozyme were prepared in $L_2O$. The pD of solutions was calculated by adding 0.40 to the reading obtained with a pH meter with a glass electrode (HM-30V; TOA Electronics Ltd.; see: Pentz, L.; Thornton, E. R. *J. Am. Chem. Soc.* **1967**, *89*, 6931−6938). The reading on the pH meter barely changed (<0.02 units) after dilution with $L_2O$ to the final concentration of 4 M $NL_4Cl$. $D_2O$ (99.9 atom % D) was purchased from Aldrich Chem. Co. $NH_4Cl$ (99.99% and 99.0% pure) was purchased from Aldrich Chem. Co. and Wako Pure Chem. Industries, Ltd. $ND_4Cl$ (98+ atom % D) was purchased from Aldrich Chem. Co., Ltd. and Acros Organics.
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