Kinetic Evidence Based on Solvent Isotope Effects for the Nonexistence of a Proton-Transfer Process in Reactions Catalyzed by a Hammerhead Ribozyme: Implication to the Double-Metal-Ion Mechanism of Catalysis

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Hammerhead ribozymes can cleave any RNA with high sequence specificity, exploiting Watson−Crick-type recognition (Figure 1a), as long as the target site contains an NUX triplet, although the efficiency of cleavage depends on the nature of the combination of N and X.[1] Since ribozymes are recognized as metalloenzymes,[2] much attention has been focused on the details of their reaction mechanisms and the involvement of Mg^{2+} ions. The first direct evidence that a Mg^{2+} ion acts as a Lewis acid by directly coordinating to the leaving 3′-oxygen was reported in the case of the ribozyme of *Tetrahymena*.[2a] Base catalysis, mediated by Mg^{2+} hydroxide, was proposed on the basis of pH−rate profiles of various metal ion-catalyzed reactions for the hammerhead ribozyme.[2f] The number of Mg^{2+} ions involved in reactions catalyzed by hammerhead ribozymes remains obscure. In order to examine whether a proton-transfer process occurs during the transition state in hammerhead ribozyme reactions, for example, by Mg^{2+} hydroxide-mediated catalysis, we measured the isotope effect of D_2O for a 32-mer ribozyme (R32, Figure 1a)[2g,3] at pH 6.0. We chose R32 because the chemical cleavage step has been unambiguously proven to be the sole rate-limiting step in this system.[3c] Therefore, all the rate constants reported in this paper represent the pure chemical cleavage step (k_{cleav} in Figure 1 of ref 3c).

Figure 2 in the supplementary material shows time courses of formation of product, followed in a solution that contained 2.5 μM ribozyme (R32), 0.5 μM substrate (R11), and 25 mM $MgCl_2$ in 50 mM MES buffer (pH 6.0) at 25 °C. All the reagents were prepared and kinetics were examined in (i) H_2O, (ii) 50% D_2O, and (iii) pure D_2O. The pH in D_2O was corrected according to the equation[4] pH = pD + 0.3139α + 0.0854α², where α is the atom fraction of deuterium and the pD is the reading obtained with a glass electrode in D_2O. Rate constants under the above single-turnover conditions were calculated by curve fitting, and they are listed in Table 1. The cleavage rate constant in H_2O ($k_{cleav}^{H_2O}$, 0.086 min^{-1}) was 4.4 ± 0.15 times larger than the corresponding value in D_2O ($k_{cleav}^{D_2O}$, 0.020 min^{-1}) at 25 °C. The rate constant in 50% D_2O ($k_{cleav}^{H_2O/D_2O}$, 0.040 min^{-1}) was an intermediate value.

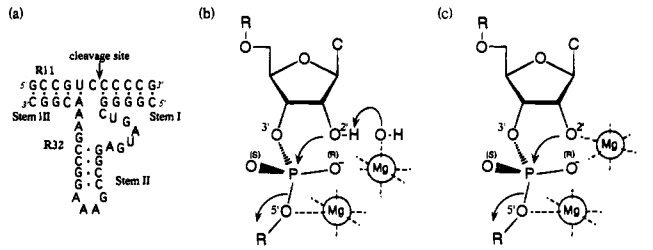

Figure 1. (a) Secondary structure of the complex of the hammerhead-type ribozyme (R32) and substrate (R11) that were used in this experiment. (b) Possible catalytic role of Mg^{2+} ions in the hammerhead ribozyme-catalyzed reaction. The reaction starts by base catalysis, possibly mediated by $Mg^{2+}OH$, which abstracts a proton from the 2′-OH of the ribose ring at the cleavage site.[2f] (c) The Mg^{2+}-bound 2′-alkoxide catalyzes the first step of the ribozyme reaction. The second Mg^{2+} ion is capable of acting as a Lewis acid catalyst by directly coordinating to the leaving 5′-oxygen. This reaction may be a concerted reaction. The *pro-R* oxygen appears to coordinate to one (or two) of the Mg^{2+} ions.[2f,14]

Table 1. Rate Constants for the Cleavage of a Phosphodiester Bond by a Ribozyme[a]

	k_{cleav} (min^{-1})	$k_{cleav}/k_{cleav}^{D_2O}$
$k_{cleav}^{H_2O}$	0.086 ± 0.003	4.4
$k_{cleav}^{H_2O/D_2O}$	0.040 ± 0.002	2.0
$k_{cleav}^{D_2O}$	0.020 ± 0.002	1.0

[a] Rate constants are averages from two sets of experiments.

The value of the apparent isotope effect of 4.4 ($k_{cleav}^{H_2O}/k_{cleav}^{D_2O}$) might be taken as evidence that supports the involvement of a proton-transfer step during the transition state. However, profiles of pH *versus* cleavage rate revealed a slope of unity in the region from pH 5.5 to pH 8.0 for the hammerhead ribozyme-catalyzed reactions.[2f,g,5] Therefore, in the range from pH 5.5 through pH 8.0, the unprotonated magnesium-containing moiety, $Mg^{2+}OH$, should be the active species.[2f,5] Since the concentration of $Mg^{2+}OD$ in D_2O is several-fold lower than that of $Mg^{2+}OH$ in H_2O,[6] the reduction of the active species, $Mg^{2+}OD$, in D_2O (ΔpK_a) could be the sole cause of the lower rate of the ribozyme-catalyzed reaction in D_2O. In order to examine this possibility, the ratio of the equilibrium constants, $K_a^{H_2O}/K_a^{D_2O}$, for $Mg^{2+}OH_2$ was estimated[6,7] at 25 °C from the linear relationship between the magnitude of the isotope effect on the acidities of various acids and their respective values of pK_a.[6−9] The estimated value ($K_a^{H_2O}/K_a^{D_2O}$) for a water molecule coordinated to a Mg^{2+} ion with a $pK_a^{H_2O}$ of 11.4 turned out to be about 4.5. Comparison of the value of $k_{cleav}^{H_2O}/k_{cleav}^{D_2O}$, namely 4.4, with the estimated value of $K_a^{H_2O}/K_a^{D_2O}$, namely 4.5,

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(4) In case of the measurements in pure D_2O, the reading from a glass electrode in D_2O (pD) was corrected to yield pH by adding a value of 0.4, according to the equation shown in the text. See: Pentz, L.; Thornton, E. R. *J. Am. Chem. Soc.* **1967**, *89*, 6931−6938.

(5) The pH−rate profile for R32/R11 in H_2O or D_2O also shows a slope of unity in the pH range from 6.0 to 8.0 (ref 2g and Sawata et al., unpublished result).

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suggests that the lower activity of ribozymes in D_2O should indeed be the result of reduced concentrations of the catalytically active species, $Mg^{2+}OH$. Therefore, the absence of kinetic isotope effects on the cleavage of phosphodiester bonds by ribozymes can be interpreted only by a mechanism in which *transfer of a proton does not take place in the transition state.*

Cleavage of RNA in general is catalyzed by acid/base catalysts in both enzymatic and nonenzymatic systems, and proton-inventory studies support the involvement of such acid/base catalysis.[10,11] In order to avoid complexities due to ΔpK_a, proton-inventory studies are usually carried out in the plateau region of pH−rate profiles. In the case of ribonuclease A (RNase A), the proton-inventory data revealed a single transition state (concerted mechanism) in which each of two protons makes a normal contribution of k^{H_2O}/k^{D_2O} of 1.75, resulting in an overall isotope effect of 3.07 in the plateau region of the pH−rate profile.[10] Similarly, in a bifunctional ribonuclease model system, in which two histidine residues act as an acid and a base catalyst, isotope effects (k^{H_2O}/k^{D_2O}) of 2.12 and 1.90 were observed for each proton in the plateau region of the pH−rate profile.[11] If a similar proton-transfer step with an isotope effect of similar magnitude had occurred in the ribozyme-catalyzed reaction, examined over a pH range where the slope of the curve was unity, the apparent isotope effect ($k_{cleav}^{H_2O}/k_{cleav}^{D_2O}$) would have been greater than 7, even with an involvement of only a single proton (naturally, two protons would double this value). An isotope effect of this magnitude could not have been missed by potential errors in our extrapolation from the linear plot.[7,8]

Ribozyme-catalyzed reactions start with base catalysis, possibly mediated by $Mg^{2+}OH$, that abstracts a proton from the 2′-OH of the ribose ring at the cleavage site (Figure 1b).[2f] Then the 2′-hydroxide attacks phosphorus to form a pentacoordinate intermediate/transition state (the first step of the RNA-cleaving reaction).[12] The second step involves the cleavage of the bond between phosphorus and the 5′-oxygen, possibly assisted by an acid catalyst. Of these two steps, our molecular orbital calculations[12] and model studies[13] indicate that it is the second step that is the overall rate-limiting step, at least in nonenzymatic reactions. Even if the first step were the rate-limiting step, a mechanism in which $Mg^{2+}OH$ abstracts the proton from the 2′-OH of the ribose ring at the rate-limiting step can be excluded because this mechanism, involving transfer of a proton at the rate-limiting step, would produce a significant isotope effect, as mentioned above. Similarly, a concerted mechanism that involves the abstraction of the 2′-OH at the transition state can be excluded if the mechanism shown in Figure 1b is the correct mechanism. Moreover, another stepwise mechanism, in which the protonation of the leaving 5′-oxygen at the second step is the overall rate-limiting step, can be excluded since such a mechanism would also produce a significant isotope effect.

Absence of major kinetic isotope effects in the ribozyme reaction can be explained if the rate-limiting step is assisted by a catalyst other than a proton. The most probable catalyst that stabilizes the leaving 5′-oxygen at the rate-limiting step (the second step) is a Mg^{2+} ion. Indeed, if a Mg^{2+} ion were to stabilize the leaving 5′-oxygen by direct coordination to it (Figure 1b), no isotope effects would be expected. Such a mechanism, in which a Mg^{2+} ion acts as a Lewis acid to stabilize the leaving group, has already been demonstrated in the case of the *Tetrahymena* ribozyme.[2a] In the case of hammerhead ribozymes, from the various pH−rate profiles in the presence of different metal ions and from thio-effects, it has been demonstrated that at least one Mg^{2+} moiety is involved in catalysis, acting as a base, and the *pro-R* oxygen appears to coordinate to the Mg^{2+} ion.[2f,14] Our data suggest that another Mg^{2+} ion, which acts as a Lewis acid, is involved in hammerhead ribozyme-catalyzed reactions. To our knowledge, this result is the first kinetic evidence that supports the possibility that a Mg^{2+} ion acts as a Lewis acid by directly coordinating with the leaving 5′-oxygen at the cleavage site of the hammerhead ribozyme.

It is possible, however, that the reaction catalyzed by a hammerhead ribozyme is a concerted process because two chemical steps such as an attack by the 2′-oxygen followed by a departure of the 5′-oxygen can be significantly catalyzed as in RNase A-catalyzed reactions. In such a concerted process, the mechanism shown in Figure 1b does not fit with our data, because the abstraction of a proton from the 2′-OH in the transition state should produce a significant isotope effect.[11] Therefore, it is likely that a more symmetrical transition state shown in Figure 1c is operative.[2g] With this mechanism, the absence of a kinetic isotope can be rationalized not only for the stepwise mechanism but also for the concerted process. Moreover, this mechanism is consistent with the principle of microscopic reversibility.

In conclusion, the absence of kinetic isotope effects supports the hypothesis that (i) transfer of a proton does not take place in the transition state and (ii) ribozymes are metalloenzymes in which one of the Mg^{2+} ions acts as the Lewis acid to stabilize the leaving 5′-oxygen.[2g,15] It is, thus, possible that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis.

Supplementary Material Available: Figure 2 showing the time courses of formation of products (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(7) It was not possible to measure the pK_a value of $Mg^{2+}(OH_2)_6$ directly by titration because multiple Mg^{2+} ions polymerize by olation in alkaline solution (pH > *ca.* 10): precipitation of the polymer hindered measurements of the correct pH value. The ratio $K_a^{H_2O}/K_a^{D_2O}$ was, thus, estimated for Mg^{2+}-OH_2 at 25 °C from the linear relationship between the magnitude of the isotope effect on the acidities of various acids and their acid strength, pK_a.[6] From the linear plots in ref 6 (see Figure 3 on page 251 in ref 6b), the ΔpK for a water molecule coordinated to a Mg^{2+} ion with a $pK_a^{H_2O}$ of 11.4 was estimated to be 0.65. Since $\log(K_a^{H_2O}/K_a^{D_2O})$, ΔpK, turned out to be 0.65, the equilibrium ratio ($K_a^{H_2O}/K_a^{D_2O}$) becomes *ca.* 4.5. Even if the accuracy of the linear plot were equivalent to a ΔpK of 0.1 (see ref 8) and, thus, the actual ΔpK for the water molecule coordinated to an Mg^{2+} ion would be 0.55 instead of 0.65, $K_a^{H_2O}/K_a^{D_2O}$ would become *ca.* 3.5. Then the actual isotope effect (k^{H_2O}/k^{D_2O}), after correction for ΔpK_a, would be 1.25, which is also significantly lower than the reported values of 3.07 and 4.03 for proton-transfer processes in RNA-cleaving reactions.[10,11]

(8) Although it was not possible to measure the pK_a of $Mg^{2+}(OH_2)_6$ directly by titration, the pK_a, in H_2O and D_2O, for a water molecule coordinated to a Cu^{2+} ion has been reported.[9] The measured pK_a values were 7.22 ± 0.03 and 7.71 ± 0.07, respectively, for $Cu^{2+}OH_2$ and Cu^{2+}-OD_2. The ΔpK estimated from the linear plot (see Figure 3 on page 251 in ref 6b) with a $pK_a^{H_2O}$ of 7.22 for $Cu^{2+}OH_2$ turned out to be 0.57; this value agrees with the measured ΔpK of 0.49 ± 0.1, within the accuracy of the experiment. This result suggests that the linear plots in ref 6 that are based on various organic acids are also applicable for estimating the ΔpK of hydrated metals.

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