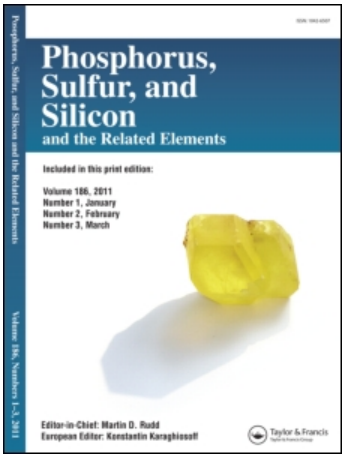

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MAGNESIUM-MEDIATED CLEAVAGE OF PHOSPHORUS-OXYGEN BOND: A RIBOZYME REACTION

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Abstract The hammerhead ribozyme belongs to the class of molecules known as antisense RNAs. However, because of short extra sequences that form the so-called catalytic loop, it can act as an enzyme. Since the catalytic domain captures Mg^{2+} ions and Mg^{2+} ions can cleave phosphodiester bonds, hammerhead ribozymes are recognized as metalloenzymes. In general, the cleavage of phosphodiester bonds involves acid/base catalysis, with proton transfer occurring in the transition state. When the possibility of such a proton-transfer process was examined by measuring solvent isotope effects, it became apparent that no proton transfer occurs in the transition state during reactions catalyzed by a hammerhead ribozyme. It is likely, therefore, that hammerhead ribozymes exploit the general double-metal-ion mechanism of catalysis, with Mg^{2+} ions coordinating directly with the attacking and leaving oxygen moieties. Moreover, NMR data suggest that Mg^{2+} ions are not only important as the true catalysts in the function of ribozyme-type metalloenzymes but they also induce the structural change in the R32 hammerhead ribozyme that is necessary for establishment of the active form of the ribozyme-substrate complex.

Key Words: RNA; NMR; ribozyme; mechanism, cleavage, magnesium.

INTRODUCTION

Naturally occurring hammerhead ribozymes were found within RNA viruses and they act "in *cis*" during viral replication by the rolling circle mechanism [1]. Hammerhead ribozymes have been engineered in such a way that they can act "in *trans*" against other RNA molecules [2,3]. The *trans*-acting hammerhead ribozyme developed by Haseloff and Gerlach [3] consists of an antisense section (stems I and III) and a catalytic domain with a flanking stem II and loop section (Fig. 1a). Because of the small size of hammerhead ribozymes, they are very suitable for mechanistic studies, being good representatives of catalytic RNA. Over the past few years, it has become apparent that ribozymes are metalloenzymes [4-11]. The first direct evidence that a Mg^{2+} ion acts as

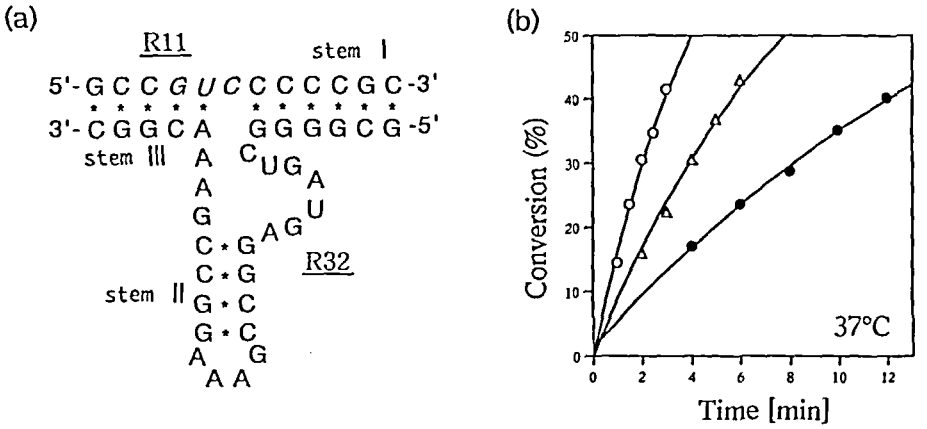

FIGURE 1 (a) *Trans*-acting hammerhead ribozyme (R32) and its substrate (R11). (b) Time courses of product formation. Reactions were followed in a solution that contained 2.5 μM ribozyme (R32), 0.5 μM substrate (R11), and 25 mM $MgCl_2$, in 50 mM MES buffer (pH 6.0) at 37 °C (single-turnover conditions). All the reagents were prepared and kinetics were examined in (i) H_2O (open circles), (ii) 50% D_2O (open triangles), or (iii) pure D_2O (closed circles).

a Lewis acid via coordination to the leaving 3'-oxygen, with stabilization of the developing negative charge on the leaving 3'-oxygen, was obtained from studies of the *Tetrahymena* ribozyme; such a mechanism was apparent from a switch in metal ion specificity with a 3'-thio-substrate [5]. Base catalysis mediated by Mg^{2+}-hydroxide was proposed on the basis of pH-rate profiles of various metal ion-catalyzed reactions of the hammerhead ribozyme [4]. Although the number of Mg^{2+} ions involved in catalysis by hammerhead ribozymes remains to be determined unequivocally, a general two-metal-ion mechanism would be well suited to a phosphotransfer reaction catalyzed by ribozymes or by protein enzymes, such as polymerases and alkaline phosphatase [8].

In the case of RNase A, a protein that is not a metalloenzyme, the reaction is initiated by a histidine residue at position 12 (His12), which acts as a base catalyst by abstracting a proton from 2'-OH (Fig. 2a). Then the resulting, more nucleophilic 2'-oxygen attacks phosphorus to generate a pentacoordinate intermediate/transition state. Finally, His119 acts as an acid catalyst by supplying a proton to the leaving 5'-oxygen, with the resultant cleavage of the exocyclic P-O(5') bond. The acid/base system

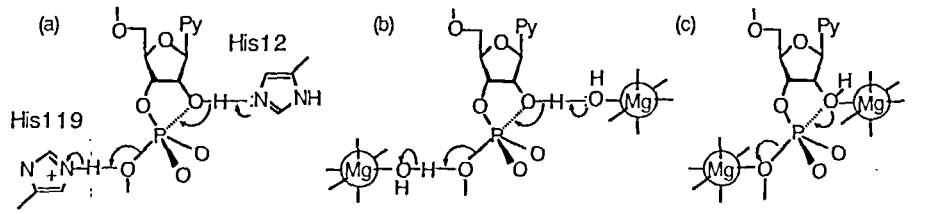

FIGURE 2 (a) Reaction mechanism for the cleavage of a phosphodiester bond by RNase A. Two histidine residues act as an acid (His119) and a base (His12) catalyst, respectively. (b) Magnesium-bound water molecules at the cleavage site of a hammerhead ribozyme catalyze the reaction by functioning as an acid and a base catalyst. (c) Catalytic magnesium ions at the cleavage site of a hammerhead ribozyme catalyze the reaction by directly coordinating to attacking and leaving oxygens. At least one magnesium ion is also directly coordinated with the *pro*-R phosphoryl oxygen.

provded by the two histidine residues in RNase A can, in principle, be replaced by Mg^{2+}-bound water moieties (Fig. 2b) [12]. Alternatively, according to our molecular orbital calculations [7,10,13-19], direct coordination of Mg^{2+} ions with the attacking or the leaving oxygen can promote formation or cleavage of the P-O bond (Fig. 2c). The latter two mechanisms [Fig. 2 (b) and (c)] should be distinguishable because the former (Fig. 2b) involves a proton- transfer process during the transition state whereas the latter (Fig. 2c) does not [11]. In order to examine whether a proton-transfer process occurs at the transition state in reactions catalyzed by hammerhead ribozymes, we measured solvent isotope effects for the 32-mer ribozyme (R32; Fig. 1a). We chose R32 because, in this case, the chemical cleavage step has been proven unambiguously to be the sole rate-limiting step in this system [11,20-23]. Moreover, in order to examine the conformational properties of the R32 ribozyme and the role of Mg^{2+} ions, we analyzed the structure by high-resolution NMR spectroscopy.

RESULTS AND DISCUSSION

In order to examine whether proton-transfer occurs during the transition state of reactions catalyzed by hammerhead ribozyme, we measured solvent isotope effects for R32 under single-turnover conditions. Figure 1b shows the time courses of product formation. All the reagents were prepared and kinetics were examined (i) in H_2O, (ii) in 50% D_2O, and (iii) in pure D_2O. The cleavage rate constant in H_2O was 3.9 times larger than the corresponding value in D_2O at 37 °C. The rate constant in 50% D_2O was intermediate between these two values. Similar effects of deuterium were observed at 25 °C [11]. Since the concentration of Mg^{2+}-OD in D_2O is several-fold lower than that of Mg^{2+}-OH in H_2O at a fixed pH, the reduction in the level of the active species, Mg^{2+}-OD, in D_2O (ΔpK_a) is the sole cause of the lower rate of the reaction in D_2O [11]. The lower activities of ribozymes (metalloenzymes) in D_2O should, therefore, be the result of reduced concentrations of the catalytically active species, Mg^{2+}-OH. Thus, the absence of any kinetic isotope effects, after the correction of ΔpK_a, in the step that leads to cleavage of phosphodiester bonds by ribozymes can be interpreted only in terms of a mechanism in which proton transfer does not take place during the transition state (Fig. 2c).

Direct proof of the mechanism, shown in Figure 2c, should be obtainable using compound E (Fig. 3). When we analyzed the stability of E, we found that the half-life of E was less than one hour at neutral pH and room temperature. E appears to be more labile than compound A, used by Cech's group [5]. In the case of E, the attack of the 2' oxygen produces F, wherein the 5' sulfur is placed at the apical position and is ready for departure. By contrast, in A, the attack of the 2' oxygen produces B wherein the 5' sulfur is at an equatorial position and, thus, unless pseudorotation takes place to produce C, the P-S bond is protected [14]. The higher reactivity of E makes it more difficult to use E in mechanistic studies.

NMR data (not shown) can be summarized as follows: 1) the R32-substrate complex cannot form without Mg^{2+} ions because the recognition arms of R32 form intramolecular base pairs (the recognition arms are closed); and 2) the addition of Mg^{2+} ions causes the recognition arms to be opened (a prerequisite for the ribozyme-substrate interaction since Mg^{2+} ions induce binding of the substrate RNA to the R32 ribozyme). Therefore, Mg^{2+} ions function not only as true catalysts but also to induce structural changes that are favorable for recognition of the substrate RNA.

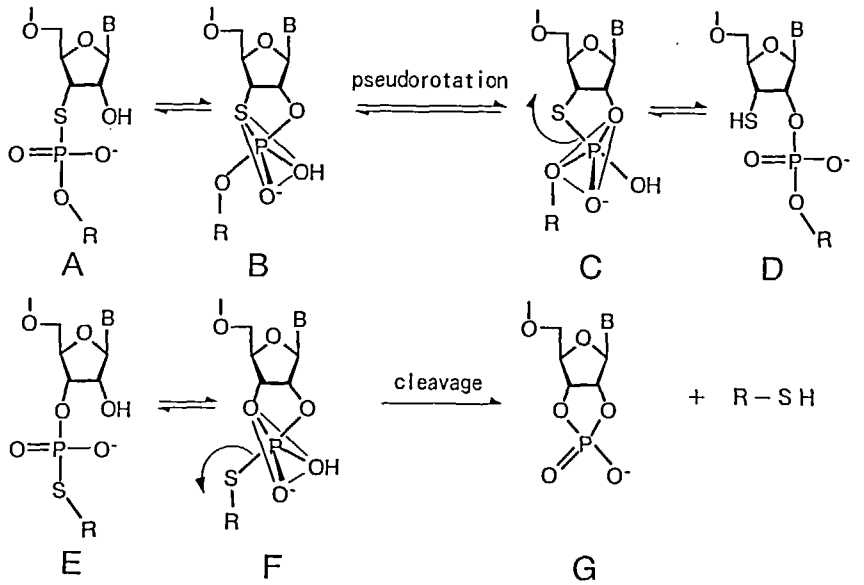

FIGURE 3 Cleavage of phosphorus-sulfur bond for 3'-thio (compound A) and 5'-thio (compound E) substituted RNA. E is more labile than A because in E the sulfur atom can be placed at the apical position without a requiremnet for pseudorotation.

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