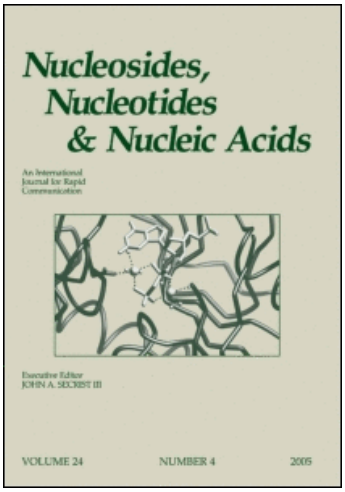

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PROPERTIES OF A HAMMERHEAD-TYPE RNA ENZYME SYSTEM THAT CONSISTS OF THREE RNA OLIGOMER STRANDS#

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Abstract: Properties of a hammerhead-type RNA enzyme system that consists of three RNA oligomers are described. The substrate 11-mer was catalytically cleaved in the presence of the enzyme components, 12-mer and 16-mer, and Mg^{2+} ions. Effects of various metal ions, Mg^{2+} concentration, and temperature on the cleavage reaction of the substrate were examined. We obtained evidence suggesting that religation of the cleaved products actually occurs. From CD titration experiments with $MgCl_2$ using non-cleavable substrates, binding constants of Mg^{2+} to the complexes were estimated.

INTRODUCTION

Among many types of RNA enzymes so far found, the hammerhead-type system is unique because an RNA oligomer system containing only about 40 nucleotide residues is fully active in the RNA cleavage reaction.[1,2] The hammerhead domain apparently contains three base-paired stems, two

#This paper is dedicated to Dr. Morio Ikehara on the occasion of his 70th birthday.

unpaired internal loops and one bulged residue (see FIG. 1). Thirteen nucleotide residues, 9 in the loops and 4 in the stem, are conserved among the hammerhead domains from natural sources. The domain can be divided into two components, the substrate and the enzyme. The substrate is catalytically cleaved in the presence of the enzyme and Mg^{2+} ions by intramolecular transesterification like the first step of ribonuclease action. This system has been extensively examined mainly by displacement of base,[2-4] sugar[5-7] and phosphate residues.[8-10] In these studies, a system that consists of two RNA strands is usually used. There are only a few reports on a system that consists of three RNA strands.[11-14]

To elucidate structure and mechanism of the hammerhead-type RNA enzymes mainly by NMR, we designed a system that consists of three RNA oligomer strands of 11, 12, and 16-mers (**1**(X=C), **3** and **2**, respectively) and contains no unnecessary terminal loops or extra dangling residues (FIG. 1). The sequences are essentially the same as those of tobacco ringspot virus satellite RNA. We chemically synthesized those oligomers in sufficient amount for NMR measurement. We also synthesized non-cleavable substrates containing 2'-O-methylcytidine (Cm) or 2'-deoxycytidine (dC) instead of cytidine (C15)[15] at the cleavage site (**1**(X=Cm) and **1**(X=dC), respectively). NMR studies on the complexes revealed that the complex indeed takes a secondary structure as expected from the hammerhead motif and the loop regions take an ordered structure even in the absence of Mg^{2+} ions.[13] $MgCl_2$ titration experiments using the non-cleavable substrates showed that Mg^{2+} binding does not cause a substantial change in the conformation of the complex and Mg^{2+} ions bind to specific regions.[14] We proposed a structural model which contains G:A mismatch base pairs between the loop residues.[14] We also showed that a 2-amino group of the third guanosine of the larger loop (G5)[15] plays an important role in maintaining the ordered structure of the loop regions and cleavage activity.[16]

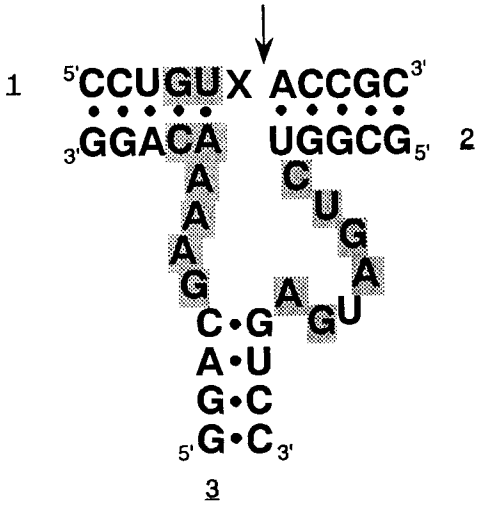

FIG. 1. Structure of the ribozyme.

In this paper, we report properties of the cleavage reaction and Mg^{2+} binding in this novel system that consists of three RNA strands.

MATERIALS AND METHODS

UV and CD spectra were measured on Shimadzu UV-2100 and JASCO J-500 spectrometers, respectively.

Synthesis of the oligoribonucleotides

$\underline{N}^4$-Benzoyl -2'-$\underline{O}$-methylcytidine was prepared by the published procedure.[16] The oligoribonucleotides were synthesized by the solid-phase phosphoramidite method using $\underline{o}$-nitrobenzyl groups for 2'-OH protection and purified as described previously.[13] The synthesis of 1(X=C and X=Cm)), 2, and 3 have been reported.[13] Isolated yield of the non-cleavable substrate containing deoxycytidine (1(X=dC)) was 115 A_{260} units (24%) starting from 5 μmol of nucleoside resin. The ε value for the modified substrates were assumed to be the same as those for 1(X=C), which was determined by a nuclease P1 digestion experiment.[13]

Cleavage reactions

The substrate was labeled by kination of the 5'-terminal OH with T4 polynucleotide kinase and [γ-^{32}P] ATP, separated by 20% polyacrylamide/ 7 M urea gel electrophoresis (PAGE) and desalted with C-18 Sep-Pak (Waters). Cleavage reaction were performed by essentially the same procedures as described by Uhlenbeck.[1] The standard reaction mixture (20 μl) contained the labeled substrate (20 pmol), ribozyme components (2 and 3, 20 pmol each), 10 mM $MgCl_2$, 50 mM tris-HCl (pH 7.5) and was incubated at 37°C for 1 h. The reaction was stopped by addition of 9 M urea, 20 mM EDTA, 90 mM tris-borate buffer (20 μl) and heating at 90°C for 5 min. The mixture was analyzed by PAGE and subsequent autoradiography.

RESULTS AND DISCUSSION

Catalytic cleavage

The substrate 1(X=C) (1μM) was cleaved to 30% and 89% in 2 min and 20 min, respectively, at 37°C in the presence of the enzyme (2+3, 1 μM) and $MgCl_2$ (10 mM). 1(X=Cm) and 1(X=dC) were not cleaved at all under the same conditions. When 50-fold excess of the substrate (50 μM) was used with respect to the enzyme, the substrate was progressively cleaved to 33% and 92% in 45 min and 3 h, respectively (FIG. 2). Thus the enzyme indeed has a catalytic activity. From these data, apparent k_{cat} and K_m can be estimated to be roughly 0.4 min^{-1} and 1 μM by Eadie-Hofstee plot of the initial cleavage rates. These values are comparable to those reported for other ribozymes.[1,7,17]

Effect of metal ions

Cleavage reactions were tried using various divalent metal ions. No cleavage was observed in the presence of Ba^{2+} or Zn^{2+} under the standard conditions (the substrate and enzyme, 1 μM each, 10 mM metal chloride, 37°C, 1 h). Mg^{2+} and Mn^{2+} ions were most effective (about 90% cleavage). Co^{2+} and Ca^{2+} ions were moderately effective (45% and 23% cleav-

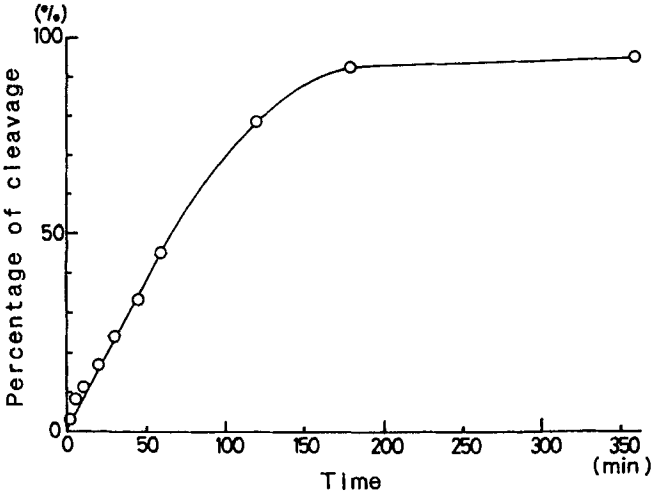

FIG. 2. Catalytic cleavage by the ribozyme. The substrate (50 μM) and enzyme (1 μM) were incubated at 37°C in 50 mM tris-HCl, 10 mM $MgCl_2$.

age, respectively). Similar efficiency of Mn^{2+}, Mg^{2+} and Ca^{2+} ions is also reported by other groups.[1,18]

Effect of Mg^{2+} concentration

The effect of $MgCl_2$ concentration (0-10 mM) was examined (FIG. 3) under the standard conditions. The cleavage yield increases almost linearly with increasing $MgCl_2$ concentration below 2 mM and reaches a plateau at around 3 mM $MgCl_2$. A similar result was obtained by Uhlenbeck.[1]

Effect of temperature

UV absorbance-temperature profiles were measured to estimate the stability of the complexes. All three complexes (about 3 μM) showed identical Tm's at around 29°C in 0.1 M NaCl, 0.01 M sodium phosphate buffer (pH 7.5). Addition of 20 mM $MgCl_2$ gave much sharper transitions for both non-cleavable complexes. Both the complexes showed identical Tm's at 42°C.

Effect of temperature on the cleavage activity was examined under the standard conditions (FIG. 4). The highest cleavage (about 90%) was observed at 20° - 40°C. When

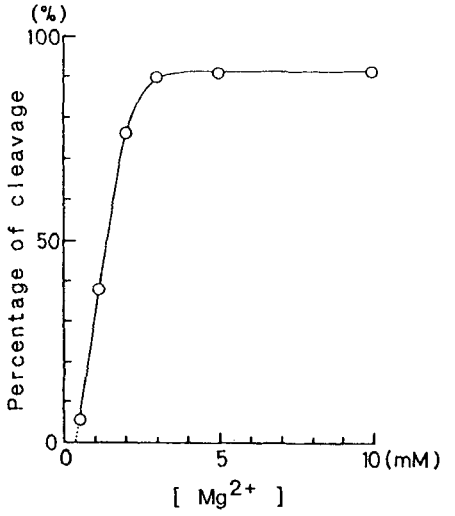

FIG. 3. Effect of $MgCl_2$ concentration on the cleavage reaction. The cleavage reaction was examined with various concentrations of $MgCl_2$ under the standard conditions (the substrate and enzyme, 1 μM each).

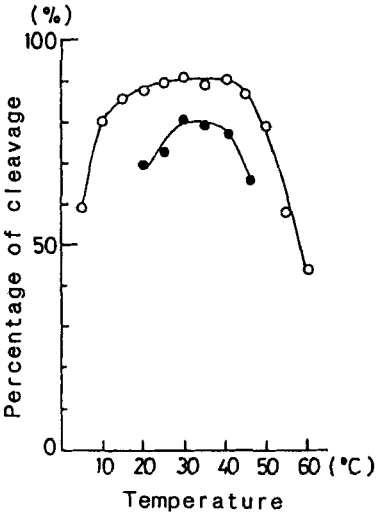

FIG. 4. The cleavage reaction was carried out at various temperature for 10 min (closed circle) or 60 min (open circle) under the standard conditions.

the reaction time was reduced to 10 min, where yields give better estimates of relative cleavage rates, the optimal temperature turned out to be between 30° and 40°C.

The above results suggest that the ribozyme is most active under the conditions where the complex is partially melted. Similar phenomena are also observed by Koizumi et al.[12] and Yang et al.[18] Their data suggest that the optimal temperature coincides with the melting temperature of the complex. It may be assumed that the active conformation is not the most stable one but some transient intermediate form is actually working as the catalyst.

Religation of the cleaved substrate

Hepatitis delta virus RNA undergoes a self-cleavage reaction in the presence of Mg^{2+} by a mechanism quite similar to that of the hammerhead ribozyme.[19] Religation of the cleaved RNA, which has newly formed 2',3'-cyclic phosphate and 5'-OH groups, occurs when Mg^{2+} is removed by addition of EDTA.[20] For the hammerhead ribozyme, it is reported that religation of monomeric tobacco ringspot virus satellite RNA (plus strand) is only detected under extreme conditions (long time incubation at 0°C with very high monomer concentration and with $ZnCl_2$) while efficient ligation was observed for the satellite RNA (minus strand)[21] which is another type of ribozyme, the hairpin ribozyme.[22]

We tried the religation reaction for our hammerhead ribozyme system. After a cleavage reaction in 12 mM $MgCl_2$, 40 mM tris-HCl (pH 8.0) for 1 h, the reaction mixture was added with 60 mM EDTA and incubated for 30 min. The extent of cleavage was 95 - 97% in the first step but it was reduced to about 90% in the second step (FIG. 5). Thus religation was observed by 7.5%, 5.5% and 4% at 23°C, 30°C and 37°C, respectively. It seems that the efficiency of religation is higher at lower temperature. These results clearly indicate a reversible nature of the ribozyme reaction. Removal of the bound Mg2+ ions by addition of EDTA may cause a conformational change of the ribozyme complex favorable for the reverse reaction, religation.

FIG. 5. Polyacrylamide (20%) gel/7 M urea electrophoresis of the cleavage reactions in 40 mM tris-HCl, 12 mM $MgCl_2$ for 60 min (lanes 2,4 and 6) and the reaction mixtures treated with 50 mM EDTA for 30 min after the cleavage reaction (lanes 3,5 and 7). Incubation temperatures were 23°C (lanes 2 and 3), 30°C (lanes 4 and 5) and 37°C (lanes 6 and 7).

Mg^{2+} titration as studied by CD

The three complexes showed almost identical CD spectra in the absence of Mg^{2+}. When the non-cleavable complexes were titrated with $MgCl_2$ (0 - 20 mM), both complexes showed an increase of the positive band at 265 nm (up to about 10%) with increasing Mg^{2+} concentration (the spectra for the Cm-complex is shown in FIG.6). If we assume that the intensity change is proportional to the fraction of Mg^{2+}-bound complex and that Mg^{2+} binding follows a simple two-state model, we can estimate the apparent number of Mg^{2+} ions bound to the complex (n) and apparent association constant (K_a = [Rz-nMg^{2+}]/[Rz][Mg^{2+}]^n;Rz: ribozyme) by the Hill plot analysis[23] (FIG. 7). It should be noted that the fraction of Mg^{2+}-bound complex vs. Mg^{2+} concentration profile (FIG. 7A) is similar to that of activity vs. Mg^{2+} concentration (FIG. 3). The latter profile seems to reach a plateau a little earlier. It may be due to that the activity shown in FIG. 3 , cleavage yield in 1 h, is not a good estimate for the relative cleavage rate.

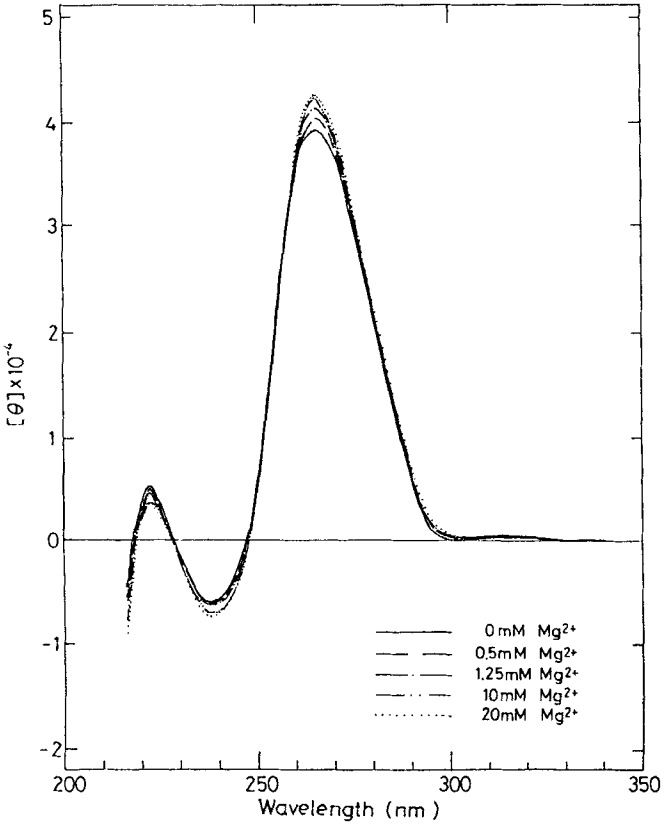

FIG. 6. CD spectra of the Cm-complex in 0. 1 M NaCl, 10 mM sodium phosphate buffer (pH 7.5) at 5°C and various concentrations of $MgCl_2$.

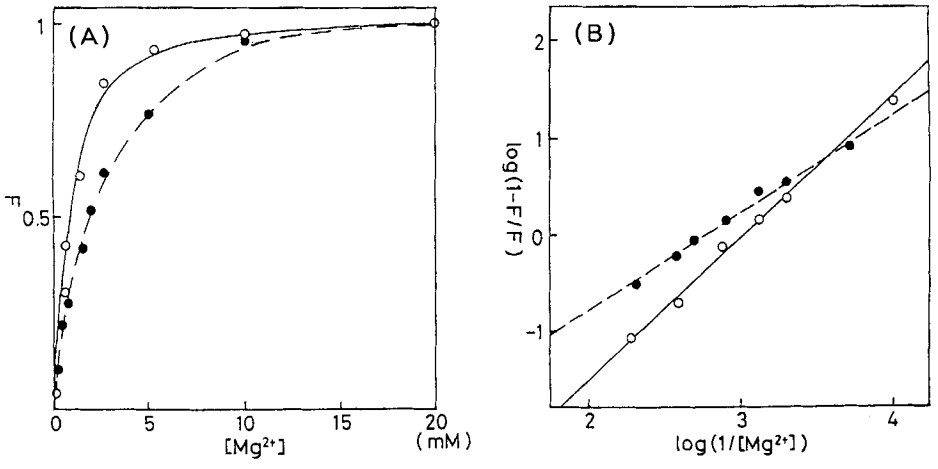

FIG. 7. (A) Profiles of fraction of Mg^{2+}-bound complex vs. $MgCl_2$ concentration for the Cm-complex (open circle) and dC-complex (closed circle). (B) Hill plot analysis of the data for the Cm-complex (open circle) and dC-complex (closed circle).

The n and K_a thus obtained were 1.46 and 2.6×10^4 for the Cm-complex and 0.97 and 5.8×10^2 for the dC-complex. These results suggest that the 2'-oxygen atom has a substantial role for Mg^{2+} ion binding to the ribozyme with respect to both the number of Mg2+ ions bound and affinity. Similar values to those of the Cm-complex are reported for another Cm-containing hammerhead ribozyme complex.[10]

In conclusion, the hammerhead ribozyme system which consists of three RNA strands shows properties quite similar to those observed for the system containing one or two RNA strands. Thus this system may be very useful for elucidation of detailed mechanism of the hammerhead ribozyme. It contains relatively short RNA oligomers which can be synthesized easily. Each oligomer can be modified separately during the synthesis to introduce a modification at a specific position for studying their effect on the function and structure of the ribozyme.

Acknowledgement

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