Magnesium-mediated Conversion of an Inactive Form of a
Hammerhead Ribozyme to an Active Complex with Its Substrate

AN INVESTIGATION BY NMR SPECTROSCOPY*

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The effects of magnesium ions on a 32-mer ribozyme (R32) were examined by high resolution NMR spectroscopy. In solution, R32 (without its substrate) consisted of a GAAA loop, stem II, a non-Watson-Crick 3-base pair duplex and a 4-base pair duplex that included a wobble G/U base pair. When an uncleavable substrate RNA (RdC11) was added to R32 without Mg2+ ions, a complex did not form between R32 and RdC11 because the substrate recognition regions of R32 formed intramolecular base pairs (the recognition arms were closed). In contrast, in the presence of Mg2+ ions, the R32-RdC11 complex was formed. Moreover, titration of mixtures of R32 and RdC11 with Mg2+ ions also induced the ribozyme-substrate interaction. Elevated concentrations (1.0 M) of monovalent Na+ ions could not induce the formation of the R32-RdC11 complex. These data suggest that Mg2+ ions are not only important as the true catalysts in the function of ribozyme-type metalloenzymes, but they also induce the structural change in R32 hammerhead ribozyme that is necessary for establishment of the active form of the ribozyme-substrate complex.

Self-degrading hammerhead RNA domains are found in many virus-like plant pathogens, and they catalyze the sequence-specific cleavage of RNA (1–3). The hammerhead ribozyme was originally predicted to consist of three base pair stems (I–III) and a central conserved nucleotide core of two nonhelical segments (Fig. 1). Many NMR studies have been performed in successful attempts to reveal the presence of these three base pair stems (4–7), but no structural information about nonhelical regions has been obtained. Recently, two crystallographic studies have provided a three-dimensional structure of the hammerhead ribozyme (8, 9). The global three-dimensional structures of these two ribozymes were nearly identical, and in these crystal structures, the base pair stems (stems I–III) that form A-type helices and the central conserved core that has two structural domains were observed; one domain of the conserved core, consisting of the sequence CUUUGAA and located next to stem I, makes a sharp turn identical to the uridine turn in transfer RNAs (10), and the other domain, consisting of conserved nucleotides adjacent to stem II, exists as a non-Watson-Crick, 3-base pair duplex (U7-G8-A9:G12-A13-A14).

A divalent cation is essential for the specific cleavage reaction of the hammerhead ribozyme, and ribozymes are recognized as metalloenzymes (11–17). The x-ray study by Scott et al. identified five potential Mg2+-binding sites in the ribozyme, one of which positioned near the catalytic pocket (9). However, the role of Mg2+ ions in the establishment of an active form of hammerhead ribozymes remains obscure, though recent electrophoretic studies demonstrated that the global conformation of the hammerhead ribozyme folds in response to the concentrations and types of ions present (18). Previously, Heus and Pardi studied the dependence of Mg2+ ions of the NMR spectrum of the ribozyme (4). However, the addition of Mg2+ ions did not cause significant spectral changes.

In order to examine the conformational properties of a 32-mer ribozyme (R32), and the further role of Mg2+ ions, we analyzed the structure by high resolution NMR spectroscopy. We chose R32 because it is a well defined ribozyme; unlike many other ribozymes, R32 does not form any inactive complexes under standard conditions for kinetic measurements (37°C, 25 mM Mg2+), a property that is required for analysis by NMR (13, 19, 20). We report here that Mg2+ ions can induce the structural change in R32 that is necessary for the interaction between the ribozyme and its substrate (RdC11).

MATERIALS AND METHODS

Preparation of the Ribozyme (R32) and of Substrate RNAs (R11 and RdC11)—The ribozyme (R32), its substrate (R11), and an uncleavable substrate (RdC11), in which the sugar of the 5′ residue at the cleavage site in the natural substrate (R11) was changed from a ribose to a deoxyribose (rC17 → dC17), were chemically synthesized on a 25-μmol scale with a 1,000 Å highly cross-linked polystyrene support on a DNA/RNA synthesizer (ABI model 3902, Applied Biosystems, Foster City, CA). The RNA phosphoramidite nucleoside monomers, A-dac, G-scf, C-isc, and U (Applied Biosystems), that we used gave rapid depuration under mild conditions. After the synthesis had been completed, the base-protecting groups were removed by heating a solution of ethanol and ammonia (1:3) that contained the oligonucleotide for 6 h at 55°C. The 2′-O-silyl groups of the base-protected oligonucleotides R11 or RdC11 were removed by treatment with a 1× solution of tetraethylammonium fluoride in THF (tetrahydrofuran). The crude product was obtained after quenching the solution with water, and it was desalted on a column of G-25 Sephadex. The silyl groups of R32 were removed with an undiluted solution of triethylamine trihydrofluoride (10 μl/OD) for 24 h at ambient temperature (21). The reaction was quenched with water, and the product was precipitated and desalted by addition of excess 1-butanol to the solution in triethylamine-tri-hy-
drofluoride. The RNA was then purified by HPLC on an anion exchange column (NucleoPac PA-100 column, 250 × 9 mm i.d., Dionex Co.) under the following gradient: starting elution with solvent A (20 mM LiClO₄, 20 mM NaOAc in H₂O:CH₃CN, 9:1, v/v, pH 6.5) and then gradually mixing with solvent B (600 mM LiClO₄, 20 mM NaOAc in H₂O:CH₃CN, 9:1, v/v, pH 6.5) to the final concentration of 70% solvent B in 40 min at a flow rate of 1.0 ml/min. The RNA obtained was desalted and isolated by the addition of four volumes of 1-propanol to the peak fraction that contained the product. Oligonucleotides were further purified by reversed-phase HPLC on a Shim-pack CLC-ODS column (Shimadzu, Kyoto). Elution was performed with a linear gradient of CH₃CN (5%-20%) in 0.1 mM TEAA (triethylamine acetate) buffer (pH 7.0). The purified oligonucleotides were desalted on a column of Sephadex G-25 (Fast Desalting column; Pharmacia Biotech Inc.). After evaporation, all counter ions were replaced with sodium by successive treatments on small columns of Dowex 50W-X2 (pyridine form), Dowex 50W-X2 (sodium form), and Chelex-100 (sodium form) resins. The fraction were collected and dried by lyophilization.

Kinetic Measurements—Kinetics of reaction were followed basically as described previously (13, 19, 20, 22). The 5’ terminus of the substrate was labeled with [γ-32P]ATP using T4 polynucleotide kinase. Reaction rates were measured in 25 mM MgCl₂ and 50 mM Tris-HCl (pH 8.0, adjusted at each temperature) under ribozyme-saturating (single-turnover) conditions at 0 or 37.5 °C. In all cases, kinetic measurements were made under such conditions that all the available ribozyme or substrate was expected to form a Michaelis-Menten complex. These conditions were achieved by employing high concentrations of the ribozyme (3.8 μM). The concentration of the substrate was 0.11 μM. The Km value of the ribozyme for its substrate was 0.02 μM at 37 °C under the present conditions (19, 20, 22). Reactions were stopped by the removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equal volume of a solution of 100 mM EDTA, 9 M urea, 0.1% xylene cyanol, and 0.1% bromphenol blue. Substrates and 5’ cleaved products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography.

Measurement of Melting Temperature of R32—In order to determine the Tm of the 4-base pair duplex and the non-Watson-Crick 3-base pair duplex regions of R32 (see “Results”), thermal denaturation of the ribozyme was monitored with a UV spectrophotometer (model 2100S, Shimadzu, Kyoto). A sample of R32 of about 1 OD/ml (total 3 ml) was prepared (i) in a phosphate buffer (pH 7.0) that contained 0.1 M NaCl and 10 mM MgCl₂. The sample was preheated at 80 °C and cooled gradually to 10 °C. The absorption of the sample at 260 nm was measured continuously at 10 °C for 10 min, and then the temperature was raised from 10 to 80 °C at a rate of 1 °C/min. The Tm was determined by plotting the second derivative of the thermal denaturation curve (not shown).

NMR Spectroscopy—All NMR experiments were performed with a model ALPHA-500 spectrometer (J. EOL, Tokyo, 500 MHz for 1H). The 1H chemical shifts were determined relative to the internal standard, 2-methyl-2-propanol (1.23 ppm). One-dimensional NMR spectra were recorded in H₂O-D₂O (4:1, v/v) that contained 0.1 M NaCl and 10 mM phosphate buffer (pH 7.0) using a 1–1 solvent suppression sequence (23). The results of nuclear Overhauser effect (NOE) experiments were recorded at 5 °C. For titration studies with RdC11, a concentrated stock solution of RdC11 was added directly to the sample tube for NMR. After each step in the titration, the sample was heated to 90 °C and slowly cooled over 30 min to allow formation of the ribozyme-oligonucleotide complex. For the titration experiments with Mg²⁺ and Na⁺ ions, concentrated solutions of MgCl₂ and NaCl were added directly to the sample tube, but the preheating to 90 °C of the oligomers was not performed because high concentration of Mg²⁺ ions can destroy RNA oligomers at high temperature.

RESULTS

Assignment of Imino Proton Resonances of R32 (Ribozyme) without RdC11 (a Pseudosubstrate) and Mg²⁺ Ions—The imino proton resonances of R32 without RdC11 and Mg²⁺ ions were assigned. In terms of its general structure, the hammerhead ribozyme consists of three base pair stems (stems I-III) and a central conserved nucleotide core of two nonhelical segments (Fig. 1). In the absence of RdC11 (a pseudosubstrate), we expected that R32 would have stem II and the GAAA loop. Fig. 2A shows the NMR spectrum of R-32 in the low field region at 5 °C. Because the imino protons can exchange with the solvent water, resonance of an imino proton is observed in the NMR spectrum only if the exchange is slow on the NMR time scale (~100 μsec). In this spectrum, many hydrogen-bonded and nonhydrogen-bonded imino protons were observed. These imino protons were assigned by one-dimensional NOE experiments (24, 25).

Stem I of R32 was identified by the sequential NOEs of the imino protons of G¹0,1, G¹0,2, G¹1,3, and G¹1,4, whose chemical shifts indicated Watson-Crick interactions between bases (Fig. 2, c and d). Because the signal observed at 10.61 ppm was associated with a NOE on the imino protons of G¹¹,4 (Fig. 2b), it was assigned to the imino proton of G¹¹,4. In Fig. 2, d and e, a NOE involving the imino proton of G¹⁰,3 and the signal at 9.88 ppm and a NOE involving the signal at 9.88 ppm and the signal at 10.13 ppm were observed. Therefore, the signals at 9.88 ppm and 10.13 ppm were assigned to the imino protons of G¹² and G¹⁰, respectively. Although the chemical shifts of these guanosine imino protons indicated that they did not form hydrogen bonds, sequential NOEs confirmed that these residues were stacked in a duplex.

In Fig. 2a, together with the imino proton signals of G¹², G¹¹, and G¹⁰ and those of stem II, some unexpected signals can be observed. NOE experiments (Fig. 2, f, g, and h) confirmed the existence of a 4-base pair duplex (G¹²-C¹³-G¹⁴-U¹⁵). This 4-base pair duplex was assigned by sequential NOEs (Fig. 2, f, g, and h). This 4-base pair duplex, including the G-U base pair, was formed between four residues in the substrate-binding region (stem III), and three of the conserved nucleotides in the catalytic loop and one nucleotide in stem I. It is well known that in a complementary double-helical oligonucleotide, a wobble G-U base pair is approximately equal to an A-U base pair in stabilizing efficiency (27, 28). Thus, an intramolecular 4-base pair duplex forms within R32.

The signal at 10.88 ppm showed no NOE, so an unambiguous assignment could not be made. It may represent imino proton of U⁹ because (i) the imino protons of G²¹,² and G²¹,², which
were predicted to exist in the flexible single-strand region (Fig. 3) are unlikely to be observed, (ii) even if they could be observed, 10.88 ppm is rather low field for the nonhydrogen-bonded imino protons of G2,2, G2,3 and G2,4, and, moreover, (iii) apart from the imino protons of G2,2, G2,3 and G2,4, all other imino protons were appropriately assigned except for U7. The complete assignments of the imino proton signals of R32, without RdC11 and Mg2+ ions, from results of one-dimensional NOE experiments are summarized in Table I.

Titration of R32 with RdC11—Fig. 4 shows the spectra of imino protons during titration of R32 with RdC11. Because the chemical shifts of protons are extremely sensitive to conformational changes, they can provide information about interactions between R32 and (pseudo)substrates. The spectra in Fig. 4 (a–f) were recorded in 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0) at 5 °C, and the spectra in Fig. 4 (g–l) were obtained under the same conditions except for the addition of Mg2+ ions. After each step in the titration, the sample tube was heated to 90 °C and slowly cooled over 30 min to induce formation of the ribozyme-substrate (R32-RdC11) complex. NMR studies of R32 without RdC11 indicated that the recognition stems of R32 formed a 4-base pair duplex (Fig. 3). In order for R32 to recognize RdC11, it is necessary for this 4-base pair duplex (G2,1-C3-U4-G5:C15,2-G15,3-G15,4-C15,5) to be opened.

Under Mg2+-free conditions, the addition of RdC11 produced additional resonances in the region between 12.5 ppm and 13.5 ppm (Fig. 4, c–f). However, the chemical shifts and the line widths of the signals from R32 did not change at all, and additional signals were observed in the spectrum of RdC11 alone without R32 (Fig. 4 a). These results indicate that R32 did not interact with RdC11 in the absence of Mg2+ ions. There are four imino protons in RdC11 (the imino protons of G1,5, U16,1, G16,2, and G16,5). However, in the spectrum of RdC11, more than four signals were observed (Fig. 4 a), including broad signals. Thus, it seemed that RdC11 adopted a random conformation and R32 existed as shown in Fig. 3. Considering that the R32 ribozyme can cleave the substrate RNA specifically without forming any

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\begin{align*}
G_{2,2} & \text{ to } G_{2,5} - 5' \\
C_{15} & - G_{1,1} \\
C_{15} & - G_{1,2} \\
A_{15} & - A_{5} \\
A_{15} & - U_{14} \\
A_{15} & - G_{6} \\
G_{15} & - A_{9} \\
G_{15} & - C_{18,1} \\
G_{15} & - C_{18,2} \\
G_{15} & - C_{18,3} \\
C_{15} & - G_{18,4} \\
G_{15} & - A_{18,1} \\
A_{15} & - A_{18,2}
\end{align*}
\]

**Fig. 3.** The proposed secondary structure of R32 in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0). Watson-Crick base pairings are denoted by solid lines, and non-Watson-Crick base pairings are denoted by outlined lines.

**Table I**

| Imino proton | Chemical shift (ppm) |
|--------------|----------------------|
| Stem I       |                      |
| G2,1         | 13.10                |
| Stem II      |                      |
| G10,1        | 12.60                |
| G10,2        | 13.01                |
| G11,3        | 12.49                |
| G11,4        | 10.45                |
| Stem III     |                      |
| G15,4        | 13.32                |
| G15,3        | 10.45                |
| GA3 loop     | 10.61                |
| Others       |                      |
| U4           | 11.64                |
| G5           | 11.94                |
| U7           | 10.89\(^{b}\)        |
| G8           | 10.13                |
| G12          | 9.88                 |

\(^{a}\) Relative to 2-methyl-2-propanol (1.23 ppm).  
\(^{b}\) This assignment is not certain.
inactive complexes at 37°C in the presence of Mg$^{2+}$ ions (13, 19, 20, 22), we were surprised to learn that even at 37°C, the recognition arms of the ribozyme were still base paired intramolecularly in the absence of Mg$^{2+}$ ions. In the many other ribozymes that have previously been studied by NMR spectroscopy (4–7), an interaction between the ribozyme and the substrate RNA can be observed without Mg$^{2+}$ ions.

In contrast to results under Mg$^{2+}$-free condition, dramatic spectral changes were observed when MgCl$_2$ was added to the solution (Fig. 4, i–l). The imino protons of U7, G8, and G12 of R32 were not observed in the presence of Mg$^{2+}$ ions (Fig. 4h). Moreover, with the addition of more and more RdC11, new broad signals appeared, and the signals due to the imino protons of G5, U4, and G15,3, which belonged to 4-base pair duplex that included the wobble G:U base pair, were gradually lost. The disappearance of signals of the other imino protons of the 4-base pair duplex (G2,1 and G15,4) was not informative because of overlapping of signals. These spectral changes can be explained by several possibilities: (i) the 4-base pair duplex of R32 was opened (the recognition arms were opened); (ii) a complex between R32 and RdC11 was formed; (iii) an equilibrium existed between the open form of R32 and the R32-RdC11 complex; and (iv) an equilibrium existed between the open form of R32, the R32-RdC11 complex, and further configuration. It is impossible to decide unambiguously among these possibilities because of line broadening and the overlapping of signals. However, it is likely that Mg$^{2+}$ ions induced the opening of the recognition arms that is necessary for the recognition of the substrate RNA. Our kinetic data indicate the formation of a ribozyme-substrate complex in the presence of Mg$^{2+}$ ions (see below).

In order to distinguish the Mg$^{2+}$-mediated complex...
formation from the charge screening salt effect, we then carried out titration experiments with Na\(^+\) ions. Fig. 5 (a and f-i) shows the titration study of mixtures of R32 and RdC11 with Na\(^+\) ions. Although the line broadening of the imino proton signals was observed with the addition of Na\(^+\) ions, the chemical shifts of these signals did not change, and no additional signals were observed. These results indicate that elevated concentrations of Na\(^+\) ions did not induce the conformational change of R32 ribozyme, and it is clear that the conformational change induced by Mg\(^{2+}\) ions originates from the essential role of Mg\(^{2+}\) ions and not from the charge screening effect.

Melting of R32—Fig. 6 shows the changes in imino proton spectra with changes in temperature. An imino proton resonance is observed only if the lifetime of the imino proton is long compared with the rate of exchange with the solvent. As the temperature is increased, base pairs begin to denature and imino protons become better able to exchange with solvent water. This phenomenon is observed in the NMR spectrum as the broadening and eventual disappearance of the signals due to imino protons. Fig. 6 shows that the imino proton signals of G\(^{10,1}\), G\(^{10,2}\), G\(^{11,3}\), and G\(^{11,4}\) (stem II) did not disappear completely even at 70 °C though all other signals were lost below 50 °C.

The thermal denaturation profile of R32 was also monitored optically (Fig. 7). One transition was observed with a melting temperature of 58 °C in the absence of Mg\(^{2+}\) ions (solid line) and with a melting temperature of 66 °C in the presence of Mg\(^{2+}\) ions (dotted line) (in both cases, calculation was made from a corresponding derivative curve; not shown). Most probably, this transition corresponded to the melting of the 4-base pair duplex and the non-Watson-Crick 3-base pair duplex. Clearly, Mg\(^{2+}\) ions contributed to the stabilization of duplexes. Melting of stem II was expected to occur at approximately 90 °C (5). However, because of line broadening above 80 °C, we did not attempt to determine the melting temperature of stem II.

Effects of the Order of Addition to the Reaction Mixture of R32, Substrate, and Mg\(^{2+}\) Ions on Rates of Reaction—Because the NMR data indicated that Mg\(^{2+}\) ions are required for establishment of the active ribozyme-substrate complex in our R32 system, we examined the effects of the order of addition of each component by measuring cleavage rates under single-turnover conditions. When Mg\(^{2+}\) ions were added last to the preincubated solution of ribozyme and substrate at 0 °C, the rate of reaction was lower than when neither ribozyme or substrate was preincubated with Mg\(^{2+}\) ions (Fig. 8). These data indicate that in the absence of Mg\(^{2+}\) ions at 0 °C, the ribozyme was trapped, at least in part, in an inactive conformation. We should add, however, that at the measurement temperature of 37 °C, the order of addition of each component did not affect the kinetic behavior (data not shown). Therefore, at the relatively high temperature of 37 °C, inactive conformers can undergo rapid conformational changes to establish the active ribozyme-substrate complex once Mg\(^{2+}\) ions are added. This phenomenon explains why we did not have to preheat R32 to 90 °C prior to our kinetic measurements (preheating did not change the kinetic behavior (13, 15, 19, 20, 22)). In other words, Mg\(^{2+}\) induces a fairly rapid transition between inactive and active folds, even without significant heating of the sample.

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**Fig. 5.** Imino proton spectra during the titration of a mixture of R32 and RdC11 (1:1) with Mg\(^{2+}\) ions in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0) at 5 °C (a–e) or with Na\(^+\) ions in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.0) at 5 °C (a, f–i). The concentrations indicated in the figure correspond to added ions (not free ions).
The Thermostable GAAA Loop in R32—Tetranucleotide loops of GNR and UNCG (where N can be any nucleotide and R can be either A or G) are known as unusually thermostable loops (30, 31), and several GNA and UNCG tetraloop hairpins have been investigated by NMR spectroscopy. The GAAA loop is a member of the GNR family, and Heus and Pardi have reported the solution structure of the GAAAtetraloop (32). In this structure, the first residue (G) and the last residue (A) in the loop form a sheared-type G:A base pair, and the phosphodiester backbone has a turn between the first residue (G) and the second residue (A). In the imino proton spectrum of R32 without both RdC11 and Mg2+ ions, the signal from the imino proton of G1 was observed at 10.61 ppm. In the study by Heus and Pardi of the GAAA loop, the signal from the imino proton of guanosine in the loop was also observed in the low field region, where signals from nonhydrogen-bonded imino protons are located (32). The rates of exchange of these imino protons with water protons should be slow, because these imino protons are thought to be buried deep inside the loop. Our NMR data confirm that a thermostable GAAA loop exists and base pairing of stem II also occurs in R32, in accord with previous studies (4, 5). These structural units were also observed in the crystal structure (8).

The Non-Watson-Crick, 3-Base Pair Duplex in R32—The x-ray studies indicated the existence of a non-Watson-Crick, 3-base pair duplex adjacent to stem II in the crystal structure (8, 9). The duplex consists of A9:G12, G8:A13, and U7:A14. A9:G12 and G8:A13 form sheared G:A base pairs (hydrogen bonds were formed between 3N of guanosine and 6NH2 of adenosine and between 2NH2 of guanosine and 7N of adenosine; Refs. 37 and 38), and U7:A14 form a non-Watson-Crick base pair (hydrogen bonds form between 6NH2 of adenosine and 2CO of uridine, and between 1N of adenosine and 2′OH of uridine).

Katahira et al. (37) studied the structure of an oligoribonucleotide that contained an adjacent G:A mismatch as a model of the hammerhead ribozyme. The sequence of the oligomer was r(GGACGAGUCC)2. Four different types of base pairing have been observed for G:A mismatches in both the crystal and solution states: (i) head to head G(anti):A(anti); (ii) sheared (side by side) G(anti):A(anti); (iii) G(anti):A(syn); and (iv) G(syn):A(anti). However, it was revealed that the 5'Py-Py-G-Pu-3'5'-Py-GAPu-3' (Py = pyrimidine, Pu = purine) sequence formed a sheared G:A base pair. This result corresponds to the results of x-ray studies (8, 9). In this model oligomer (37), the signal from the imino proton of the guanosine in the sheared G:A base pair was observed at 10.29 ppm, because the rate of exchange with water protons is restricted by the neighboring base pairs, in spite of the absence of hydrogen bonding. In the NMR spectrum of R32, the signals from the imino protons of G12 and G8, as well as data that support the existence of sheared G:A base pairs in the ribozyme, have not previously been published.
We assigned the signal at 10.88 ppm to the imino proton of U7, although no apparent sequential NOE could be detected (see "Results"). In the x-ray crystallographic structure, U7 forms a non-Watson-Crick base pair with A14, and hydrogen bonds are formed between 6NH2 of adenosine and 2CO of uridine and between 1N of adenosine and 2′OH of uridine, whereas the imino proton of U7 is involved in the hydrogen bonding to O6 of G8, the 3′-neighboring residue (8). We could not clarify the base-base interaction between U7 and A14, but the presence of a signal at 10.88 ppm in the NMR spectrum suggests that U7 and A14 do not form a Watson-Crick base pair and that the rate of exchange of the imino proton of U7 with water protons is low. This result is not inconsistent with the non-Watson-Crick A14-U7 base pair in the x-ray structure.

4-Base Pair Duplex Including a G:U Base Pair in R32—One-dimensional NOE experiments suggested that G15,2-C15,3-U15,4-G15,5 and C15,2-G15,3-G15,4-C15,5 in R32 formed a base pair duplex that included a wobble G:U base pair (Fig. 3). Previously, Heus and Pardi studied the substrate-free ribozyme by NMR spectroscopy (4), but neither a 4-base pair duplex that included a wobble G:U base pair nor a 3-base pair non-Watson-Crick duplex that followed stem II were observed. Because the sequence of their ribozyme in the region that corresponded to 4-base pair duplex in R32 was not complementary, in the case that they examined, the 4-base pair duplex did not form. Moreover, because this 4-base pair duplex would be expected to stabilize the 3-base pair non-Watson-Crick duplex, this region was probably more flexible in the ribozyme studied by Heus and Pardi (4). This difference probably explains why a 4-base pair duplex and a 3-base pair non-Watson-Crick duplex were observed only in the R32 ribozyme.

Our proposed secondary structure of the R32 (ribozyme) without RdC11 and Mg2+ ions is shown in Fig. 3. In this model, there are seven Watson-Crick G:C base pairs, three sheared G:A base pairs, one wobble G:U base pair, and one non-Watson-Crick A14-U7 base pair. We could not clarify the base-base interaction between A6 and A15,1, because neither residue has an imino proton and the base protons of A6 and A15,1 could not be assigned. However, it is likely that the adenine rings of A6 and A15,1 stack in the duplex and adopt a base pair-like configuration, because an A:A base pair has been observed in several RNAs (40, 41). If the A6-A15,1 base pair exists, R32 would have a long duplex with 13 continuous base pairs. Existence of the 4-base pair duplex and the 3-base pair non-Watson-Crick duplex may reflect the observed melting temperatures of 58 and 66°C, respectively, in the absence and the presence of Mg2+ ions (Fig. 7).

The Effect of Mg2+ Ions on R32—The specific cleavage reaction of the hammerhead ribozyme requires a divalent cation and ribozymes are recognized as metalloenzymes (11–17). X-ray studies by McKay’s group showed that a strong coordination site for a metal ion exists in the non-Watson-Crick 3-base pair duplex of the central core (the N7 atom of G101 and the pro-Rp oxygen of the phosphate of A90) (8), and Klug’s group also identified this metal binding site (9). Our experiments showed that the signals from the imino protons of G12, G8, and U7 disappeared upon the addition of Mg2+ ions (see Fig. 4, b and h). Although the imino proton signal of G8 of R32 in the absence of RdC11 did not disappear upon the addition of Mg2+ ions (see Fig. 4, b and h), the imino proton signals of G8 and those of the neighboring residue, U4, were lost upon the addition of Mg2+ ions in the presence of the uncleavable substrate, RdC11 (see Fig. 5). These results may support the existence of the strong binding site for a divalent ion indicated by the crystallographic studies (8, 9). Alternatively, Mg2+ ions may function to destabilize the duplex formed by the residues of the central core. Unfortunately, our analysis could not identify the exact binding site of the catalytic Mg2+ ion in the R32 ribozyme system, as previous NMR studies could not (4–7, 39). A further examination is required toward a detailed structural characterization or toward locating the site of metal binding, for instance by using Mn2+ (if it does fold the RNA in a similar way to Mg2+) as a paramagnetic probe.

Our titration studies suggested that the R32-RdC11 complex was not formed in the absence of Mg2+ ions and that Mg2+ ions provided the properties in R32 necessary for the ribozyme-substrate interaction (Figs. 4 and 5). Titration studies of ribozymes by other groups indicated that Mg2+ ions did not induce essential conformational changes in ribozymes (4, 7). However, there is a significant difference between such ribozymes and our R32 ribozyme. The ribozymes previously studied formed a complex with the substrate RNA without Mg2+ ions. Under the same conditions, R32 cannot form a complex with the substrate RNA (RdC11), an event that is prerequisite for the specific cleavage by R32 with Mg2+ ions. Due to the existence of the unfavorable conformation within the R32 ribozyme, we could detect an addi-
tional structural role of Mg$^{2+}$ ions, properties that could not be detected in previous studies (4, 7).

Generally, Mg$^{2+}$ ions have a function to facilitate the intermolecular duplex formation because of the charge screening effect. Therefore, our finding that Mg$^{2+}$ ions induce the interaction between the ribozyme (R32) and the substrate (RdC11) may be taken as one of those examples. However, our NMR studies indicated that this intermolecular interaction was not induced by elevated concentrations of Na$^+$ ions (even in the presence of 1.0 M NaCl) (Fig. 5, a and f-i). Therefore, it is clear that the conformational change of our ribozyme was not caused by the charge screening effect and it is due to the essential role of Mg$^{2+}$ ions.

Because our R32 ribozyme forms the intramolecular base pairs that could not recognize the substrate RNA, it may be thought that our experiment is a particular system. However, many ribozymes suffered from formation of inactive structures, resulting in incomplete cleavage of substrates. Ribozymes studied by Sarma et al. (39) also showed intramolecular base pairs in the absence of Mg$^{2+}$ ions, although they also catalyzed the sequence-specific cleavage of the substrate RNA. Many other ribozymes expressed in vivo have a higher chance to form intramolecular base pairs because of their extra flanking sequences, leading to reduced catalytic activities (42–46). Therefore, the intramolecular base pairing is not particular to our R32 ribozyme but rather is general for many ribozymes. In fact, in terms of its kinetic behavior, our R32 ribozyme is one of the better ones: there has been no indication of formation of any inactive structure of R32 under standard conditions for kinetic measurements (in the presence of 25 mM Mg$^{2+}$ ions). Lilley's group clearly demonstrated global conformational changes of the hammerhead ribozyme in response to the concentrations of Mg$^{2+}$ ions (18). Our finding complements that of Lilley's group: if a ribozyme forms an unfavorable conformation, Mg$^{2+}$ ions might help establish correct a ribozyme-substrate complex especially for trans-acting ribozymes.

Fig. 9 shows a schematic representation of the proposed effect of Mg$^{2+}$ ions on the R32 ribozyme. Under Mg$^{2+}$-free conditions, R32 does not interact with RdC11 because the substrate recognition region of R32 forms intramolecular base pairs. The binding of Mg$^{2+}$ ions to the central conserved nucleotide core of the R32 ribozyme induces a conformational change in R32 and, probably, the substrate recognition regions of R32 can now interact with RdC11. Although the exact binding site of the divalent ion was not revealed, it could involve the region of the 3-base pair U$^\gamma$-G$^\alpha$-A$^\omega$, U$^\gamma$-G$^\alpha$-A$^\omega$, and A$^\omega$-A$^{14}$ duplex because signals from the region disappeared upon addition of Mg$^{2+}$ ions. It is well known that a divalent cation is essential for the specific cleavage reaction of the ribozyme, but it was not previously clear whether the catalytic Mg$^{2+}$ ions stabilize the tertiary structure of the active form of the ribozyme. In the cleavage reaction of the R32 ribozyme, at least, Mg$^{2+}$ ions act to induce changes in its property that are favorable for recognition of the substrate RNA. It remains to be determined whether the same Mg$^{2+}$ ions have a catalytic as well as a structural function.

Conclusion—Our data can be summarized as follows: (i) the secondary structure of R32 in the absence of Mg$^{2+}$ ions is as shown in Fig. 3; (ii) the R32-RdC11 complex cannot form without Mg$^{2+}$ ions because the recognition arms of R32 form intramolecular base pairs (the recognition arms are closed); and (iii) the addition of Mg$^{2+}$ ions (but not Na$^+$ ions) causes the recognition arms to be opened (a prerequisite for the ribozyme-substrate interaction because Mg$^{2+}$ ions induce binding of the substrate RNA to the R32 ribozyme). Mg$^{2+}$ ions were thought previously to play an integral role in catalytic function (13, 15) and not in structural stability, and the folding of the ribozyme that is dependent on Mg$^{2+}$ ions has not been observed in previous NMR studies (4, 7). Recently, electrophoretic studies demonstrated that the ribozyme folds in response to the concentration and type of ion present (18). Mg$^{2+}$ ions induced co-linear alignment of stems II and III with a variable angle subtended by stem I that depended on the concentration of Mg$^{2+}$ ions. Moreover, the proposed Mg$^{2+}$-binding site (18) may explain the disappearance of our signals from the 3-base pair U$^\gamma$-G$^\alpha$-A$^{14}$ duplex. In this report, we showed that Mg$^{2+}$ ions function to induce structural changes that are favorable for recognition of the substrate RNA. This is the first NMR study to indicate that the structural change in a ribozyme that is required for substrate recognition can be induced by Mg$^{2+}$ ions.
Magnesium-mediated Conversion of an Inactive Form of a Hammerhead Ribozyme to an Active Complex with Its Substrate: AN INVESTIGATION BY NMR SPECTROSCOPY
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