The Kinetics and Magnesium Requirements for the Folding of Antigenomic δ Ribozymes

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Using an oligonucleotide hybridization assay to gain insight into the folding of δ ribozymes, we demonstrate a correlation between their folding and catalytic behavior. Together with recent structural information on the crystal structure of self-cleaved genomic δ ribozyme, in which the L3 loop interacts with J1/4 to form the newly proposed stem P1.1, we conclude that it is likely that the P1.1 stem forms only in the presence of Mg^{2+}. This stem can be detected in both the self-cleaved and *trans*-acting δ ribozymes. When the *trans*-acting version of antigenomic δ ribozyme was studied, it is demonstrated that its L3 loop requires magnesium and, apparently, formation of the P1 stem for the subsequently formation of the P1.1 stem. Most importantly, the kinetics were monitored, and provide a significant addition to our understanding of ribozyme tertiary structure formation prior to the chemical cleavage step. Using previous kinetic data and our new findings, we discuss the rate-limiting characteristics of δ ribozyme folding. © 2000 Academic Press

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The folding mechanisms of macromolecules, such as proteins and RNAs, have been used to define their structure-function relationships. The folding of a protein into its functional conformation relies on a large array of functional groups located on the side chains of its amino acid monomers. In contrast, folding of an RNA molecule is restricted to a repertoire of interactions involving only highly charged phosphodiester bonds, the ribose moiety and the functional groups of four nucleotide bases (1). Consequently, RNA molecules rely on surrounding molecules such as metal ions, proteins, and water, to assume their functional tertiary conformations. Over the past few years there

has been considerable progress in our understanding of the kinetics of RNA folding due to simultaneous advances in experimental and theoretical methods (for a review, see Ref. 2). These advances, which include ribonuclease mapping, UV cross-linking, induced cleavage, chemical interference and mutational analyses, have been used to define the possible conformations of various RNA molecules (2). Most significantly, advanced techniques of crystallography, X-ray diffraction and NMR have precisely displayed several RNA structures. Currently, the structure-function relationship of group I intron derived ribozymes is the most thoroughly investigated. Several of the approaches used to study this large ribozyme have also been applied to smaller ribozymes, the best studied of which is the hammerhead ribozyme. However, several limitations have been encountered; for example, some crystal structures of hammerhead ribozymes could not be used to define transition state conformations (3–6).

Experimental approaches permitting the simultaneous survey of the spatial arrangement of both the fully active and the transition states of catalytic RNAs are likely to be of great value in defining the structure-function relationships. For instance, real-time fluorescence resonance energy transfer (FRET) has been used to investigate the structure of fully active hammerhead and hairpin ribozymes, as well as isolated subdomains of these, and has provided more precise information on the folding kinetics of these ribozymes (for example see 7, 8).

The oligonucleotide hybridization assay has been successfully used in RNA folding studies, most notably in elucidating the folding pathways of large ribozymes including the group I ribozymes and RNase P (9, 10). Based on the fact that no *a priori* knowledge about the structure of the RNA molecule in question is required, the oligonucleotide hybridization assay therefore offers some distinct advantages over FRET. The former method relies on the following two premises: (i) the selective susceptibility of RNA:DNA hybrids to RNase H cleavage, and (ii) the accessibility of DNA oligonu-

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cleotides to various exposed single-stranded domains on the RNA molecules to be investigated (9). Either under partially denaturing or native conditions, in which oligonucleotide hybridization and the resuming of native structure are rival determinants, the RNase H activity is used to determine the accessibility of a target area on the RNA molecules as a function of time (Fig. 1).

In this study, we have adapted the oligonucleotide hybridization assay in order to study the folding kinetics of δ ribozymes (Figs. 1 and 2). The δ ribozymes were originally identified as self-cleaving motifs within the single-stranded RNA genome of hepatitis δ virus. Recently, the crystal structure of a genomic self-cleaved δ ribozyme was described as being a tight structure possessing an additional, previously unidentified, stem (P1.1) generated through the interaction of L3 loop and J1/4 nucleotides (11). Wadkins *et al.* (12) subsequently mutagenized the positions required for the formation of the P1.1 stem and showed that these base-pairing interactions are essential for the cleavage activity of self-cleaving δ ribozyme. Nishikawa and Nishikawa (13) recently used an *in vitro* selection protocol to demonstrate that magnesium ions are likely to play an important role in the P1.1 stem formation. Unlike hammerhead and hairpin ribozymes, δ ribozyme cleavage is not supported by monovalent ions (14). The catalytic activity of self-cleaving δ ribozymes was observed to vary with both the presence of denaturing agents and temperature, suggesting a conformational requirement (15, 16); however, no alternate conformations were detected by native polyacrylamide gel electrophoresis (17, 18). It has recently been demonstrated that imidazole can be utilized as a general base in the chemical cleavage step of δ ribozymes (19). Consequently, the divalent metal ion requirement of δ ribozymes might be essential for the folding of the ribozyme. Clearly it is of interest to use a more powerful method to characterize the folding of δ ribozymes, whose catalytic behavior is unique among ribozymes. In this report the folding kinetics of both *cis-* and *trans*-acting versions of antigenomic δ ribozymes are examined using oligonucleotide hybridization assay. We show that Mg^{2+} is involved in the folding of δ ribozymes, and discuss whether or not the folding of δ ribozyme is the rate limiting step in its catalytic pathway.

MATERIALS AND METHODS

RNAs. Ribozymes and RNA substrates were synthesized, purified and ^{32}P-end-labeled as described previously (18). Two forms of antigenomic δ ribozymes, a *trans*-acting ribozyme and a *cis*-acting ribozyme, were used in this study. Their sequences and structures are depicted in Fig. 2A. These ribozymes are referred to as *trans*-δ-Rz and *cis*-δ-Rz, respectively. Following *in vitro* transcription and self-cleavage of *cis*-δ-Rz, the resulting cleavage product was purified on a

10% denaturing PAGE gel. An oligoribonucleotide (RNA-P2) and an RNA-mixed polymer (SdC4) with a deoxyribose residue substituted for the ribose one at position 4, corresponding to the 3′-cleavage product and the substrate, respectively, were synthesized (Keck Oligonucleotide Synthesis Facility, Yale University).
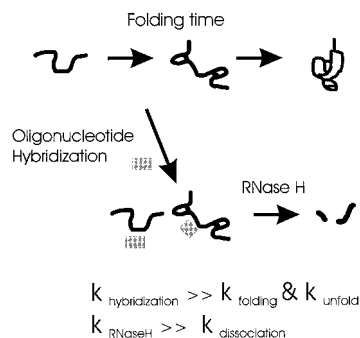
Oligonucleotides. Oligonucleotides were synthesized, deprotected, and desalted by Gibco BRL Custom Primer Service (Canadian Life Technologies Inc.). The sequences of the oligonucleotides, corresponding to the stems (P1 and P4), junctions (J1/4 and J4/2) and loops (L3 and L4) of antigenomic δ ribozymes are illustrated in Fig. 2A, and are *L3*, 5′-GCGAGGA-3′; *P1*, 5′-AGGTCGG-3′; *J1/4*, 5′-CATGCCC-3′; *P4*, 5′-CCGAAGG-3′; and *J4/2*, 5′-CCTTAGC-3′. Italic letters were used to identify the oligonucleotides.

Buffers. RNA dissolving buffer is DEPC-treated water containing 1 mM Tris–HCl, pH 8.0 and 0.1 mM EDTA. Folding buffer (10×) contained 500 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM dithiothreitol and various concentrations of $MgCl_2$ ranging from 0 to 200 mM. RNase H cleavage buffer (10×) was identical to folding buffer except that it contained various concentrations of the oligonucleotides and the required amount of $MgCl_2$ such that the final concentration of $MgCl_2$ in the RNase H cleavage reactions was 10 mM. Enzyme assay buffer (10×) was identical to folding buffer except that it contained 100 mM $MgCl_2$. Reaction stop solution contained 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol in 95% formamide.

Oligonucleotide hybridization assays. End-labeled ribozyme in RNA dissolving buffer (6 μl) was denatured at 95°C for 1 min. Ribozyme folding and oligonucleotide annealing were initiated by the addition of buffer containing varying $MgCl_2$ concentrations, and then lowering the reaction temperature to an endpoint of 37°C. A Perkin–Elmer DNA Thermal Cycler 480 was used to obtain a constant rate of temperature change of 1°C/s. An RNase H (0.15 units/μl, United State Biochemical) cleavage mixture (6 μl) that had been preincubated at 37°C for 2 min was then added at various time intervals. RNase H cleavage was allowed to proceed for 30 s at 37°C, and then arrested by the addition of ice-cold reaction stop solution (6 μl). The reaction mixtures were kept on ice until fractionation on 10% denaturing PAGE gels. The gels were then exposed to phosphorimaging screens (Molecular Dynamics).

RESULTS AND DISCUSSION

We have characterized the folding of δ ribozymes using the oligonucleotide hybridization assay previously described by Zarrinkar and Williamson for the group I intron ribozyme (9). Based on (i) the differential accessibility of short oligonucleotides to regions located on the RNA molecules, and (ii) the high specificity of RNase H to cleave newly formed DNA:RNA hybrids, the fraction of the RNA population that progressively folds following the addition of metal ions can be monitored in a time course experiment (see the schematic representation depicted in Fig. 1). In order to have a valid assessment of δ folding we designed oligonucleotides, ranging between 5 to 10 nt long, that corresponded to the single-stranded regions of *trans*-δ-Rz (i.e., L3 loop, P1 helix, L4 loop, J1/4 and J4/2 helices, Fig. 2A). The 7-nt long oligonucleotides were found to give the highest specificity for the particular δ ribozyme used in this assay. Preliminary experiments testing the ability of these oligonucleotides to drive the

FIG. 1. Oligonucleotide hybridization assay. A schematic representation of the assay of *trans*-acting δ ribozyme in the absence of its substrate using the method of Zarrinkar and Williamson (9). As the partially denatured RNA molecules progressively fold into their native structures, some nucleotides or areas gradually participate or interact with other nucleotides, thereby enabling the molecule to resume their native conformation. As a result these areas become inaccessible to the oligonucleotide, and therefore are resistant to subsequent cleavage by RNase H.

RNase H digestion of *trans*-δ-Rz are shown in Fig. 2B. When oligonucleotides *L3* and *P1* (0.5 μM) were used under conditions in which *trans*-δ-Rz (1 nM) was partially denatured in a solution containing 1 mM Tris–HCl, pH 8.0, and 0.1 mM EDTA, specific RNase H derived products were detected (Fig. 2B, lanes 2 and 3). Oligonucleotides *J1/4* and *P4* yielded no RNase H digestion products (Fig 2B, lanes 4 and 5), indicating that the J1/4 helix and P4 hairpin-loop of *trans*-δ-Rz are likely concealed in the tight complex structure. Another possible explanation is that the hybridization of oligonucleotides *J1/4* and *P4* is either inefficient or sterically hidden. In addition, oligonucleotide *J4/2* gave both the expected product and a nonspecific product following RNase H cleavage (Fig. 2B, lane 6). Based on the specificity of the oligonucleotide binding and RNase H assay, only oligonucleotides *L3* and *P1* were used in the subsequent studies.

In order to ensure that the annealing of oligonucleotides *L3* and *P1,* and of RNase H cleavage, occurred at a rate superior to that of the RNA folding on itself, various concentrations of oligonucleotides (0.5 to 10 μM) and RNase H (0.01 to 0.5 units/μl) were tested against a trace amount of RNA (1 nM). It is crucial to ensure that no folding takes place subsequent to the addition of the oligonucleotides and RNase H so that the RNase H cleavage products truly reflect the nature of RNAs at the time when samples were taken and subjected to the assay. We found that at a final RNase H concentration of 0.15 units/μl, RNase H cleavage is dependent on the oligonucleotide concentration; and that at oligonucleotide concentrations of 5 to 10 μM, greater than 90 percent of the RNA substrate was cleaved in less than 30 s (no graphical data shown). Thus, the conditions which will allow us to accurately
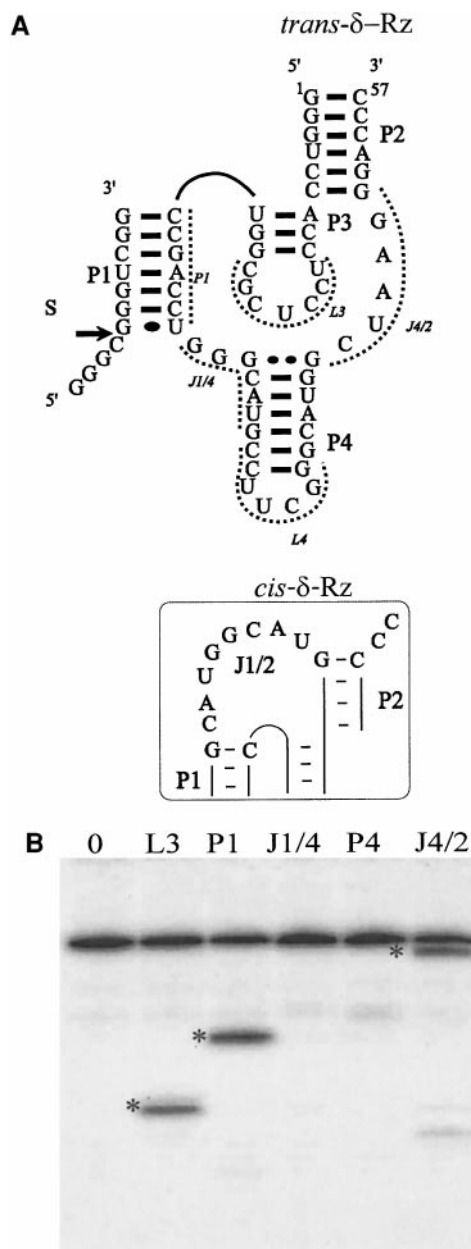
FIG. 2. (A) Sequences and predicted secondary structures of *trans*- and *cis*-acting antigenomic δ ribozymes. *trans*-δ-Rz consists of 57 nt., its substrate (S) 11 nt., and *cis*-δ-Rz 63 nt. The predicted structures are based on Perrotta and Been (26). The P1, P2, P3, and P4 stems are indicated in bold. Junction J1/2, which joins stems P1 and P2, was reconstructed from *trans*-δ-Rz to produce the *cis*-δ-Rz used in this study. The inset shows the difference between *cis*- and *trans*-δ-Rz. The dotted lines represent regions targeted for hybridization assays, with the corresponding probes indicated in italics (*L3, P1, J1/4, L4* and *J4/2*). (B) Autoradiogram showing the products of the probe hybridization and RNase H cleavage on *trans*-δ-Rz in the preliminary assays. The ribozyme in a solution containing 1 mM Tris-HCl pH 8.0 and 0.1 mM EDTA was subjected to oligonucleotide hybridization assay as described in Materials and Methods. The first lane is a control containing no oligonucleotide. The stars indicate the expected products.

examine the δ ribozyme folding contain a trace amount of ribozyme (1 nM) in RNA dissolving buffer, oligonucleotide (5 μM), RNase H (0.15 units/μl), and various concentration of MgCl$_2$ (0.01 to 50 mM) to induce RNA folding. Under these conditions rapid hybridization and cleavage rates were obtained, and the time course experiment can be performed in order to monitor the progression of δ ribozyme folding in solution using calculated values of the fractions of folded (full length δ-Rz left) and unfolded (RNase H digested products) δ-Rz.

The δ Ribozyme Folding

Under the conditions allowing accurate measurement of oligonucleotide hybridization and RNase H cleavage, we first monitored the folding status of δ-Rz molecules under partially denaturing and native conditions. End labeled *cis-* or *trans-*δ-Rz (1 nM) was dissolved in RNA dissolving buffer (1 mM Tris-HCl pH 8.0/0.1 mM EDTA) and heated to 95°C so as to completely denature it. The proportion of folded and unfolded molecules was measured under partially denaturing conditions in which the completely denatured Rz molecules were allowed to resume their natural state in solution containing 50 mM Tris pH 8.0, 10 mM NaCl, 0.01 mM EDTA, and 1 mM dithiothreitol (i.e. a buffered solution without divalent metal ions) for 5 min prior to being subjected to oligonucleotide hybridization and RNase H cleavage assays. For the experiments under native conditions, the completely denatured ribozymes were incubated in the above buffer containing 10 mM MgCl$_2$ prior to oligonucleotide hybridization and RNase H cleavage assays. Using oligonucleotides *P1* and *L3,* the folding of both *cis* and *trans-*δ-Rz was monitored. It is important to note that when less RNase H cleavage products were detected at the accessible P1 helix or L3 loop, it indicates at least two possibilities. First, the target helix or loop might be inaccessible to the oligonucleotide hybridization in the folded structure. Secondly, the target nucleotides or areas might participate in other types of interactions (i.e., base-paring or other tertiary interactions). In order to avoid any confusion in using various terms describing the lack of RNase H cleavage, the term "folded" was used when either no or less RNase H products were detected, and the term "unfolded" was used when the helix and loop were accessible or cleaved in the assays.

P1 helix folding. Based on the predicted secondary structure of *delta* ribozymes, the P1 helix is a substrate binding site which could bind oligonucleotide *P1* (Fig. 2A). Approximately 80% *trans-*δ-Rz (1 nM) under partially denaturing conditions was found to be unfolded as it was digested in the oligonucleotide *P1* binding-RNase H cleavage assay (Fig. 3A, hashed bar). When
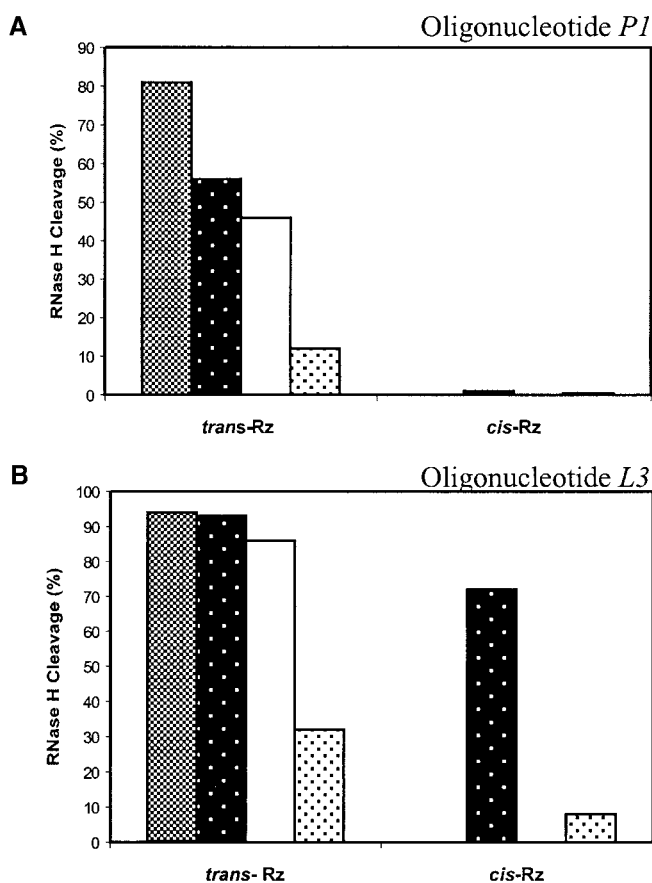
FIG. 3. Folding of the P1 helix and L3 loop. (A) Histogram depicting the quantitative analysis of the oligonucleotide *P1* hybridization assay. *Trans-*δ-Rz was incubated under partially denaturing or native conditions, in the absence or presence of the cognate substrate. Self-cleaved *cis-*δ-Rz product was used as a control to illustrate the inaccessibility of the P1 domain. (B) Histogram depicting the quantitative analysis of the oligonucleotide *L3* hybridization assay on both *trans-*δ-Rz and the self-cleaved product of *cis-*δ-Rz. For both panels, the hashed bars represent partially denaturing conditions in which no substrate was added. The white dotted black bars represent native conditions in the absence of the cognate substrate. Clear bars represent denaturing conditions in the presence of the cognate substrate. The black dotted white bars represents native conditions under which *trans-*δ-Rz and either its substrate or self-cleaved *cis-*δ-Rz were assayed.

an excess amount of the substrate (100 nM) was incubated with a trace amount of *trans-*δ-Rz (1 nM) under partially denaturing conditions, approximately 60% of *trans-*δ-Rz remained unfolded and was digested (Fig. 3A, dotted black bar). The decrease in the accessibility of the P1 helix is likely due to substrate and oligonucleotide *P1* competition. This finding supports the notion that the base-pairing interaction of substrate and ribozyme takes place in the absence of divalent metal ions as observed during our investigations on the optimization of reaction initiation to yield maximum catalytic cleavage. Our studies (data not shown) and pre-

vious report by Fauzi *et al.* (20) demonstrated that catalytic assays would give maximum cleavage when the substrate and ribozyme were primarily annealed in the absence of divalent ions. Under native conditions, approximately 50% of *trans*-δ-Rz molecules remained unfolded and was digested by the oligonucleotide *P1* driven RNase H (Fig. 3A, clear bar). This finding indicates that *trans*-δ-Rz in solution is a heterogeneous mixture. When *trans*-δ-Rz and its cognate substrate were incubated under native conditions, 10% of *trans*-δ-Rz remained unfolded and was cleaved as a result of oligonucleotide *P1* driven RNase H activity (dotted white bar), indicating that a small fraction of *trans*-δ-Rz was either not able to bind the cognate substrate, or was not sensitive to the magnesium induced folding. This finding strongly suggests the presence of a ribozyme conformer that is unable to form the ribozyme-substrate complex. Similar experiments were conducted for *cis*-δ-Rz. Under both partially denaturing and native conditions, the P1 helix of *cis*-δ-Rz was completely protected from oligonucleotide binding and RNase H cleavage (Fig. 3A).

L3 loop folding. Under partially denaturing conditions, approximately 90% of *trans*-δ-Rz (1 nM) remained unfolded and was cleaved in the oligonucleotide *L3* hybridization and RNase H cleavage assays (Fig. 3B, hatched bar). The presence of an excess amount of substrate (100 nM) under partially denaturing conditions did not change the high accessibility of L3 loop in *trans*-δ-Rz, since 90% of *trans*-δ-Rz was remained unfolded and was digested by RNase H (Fig. 3B, dotted black bar). Under native conditions, approximately 90% of *trans*-δ-Rz remained unfolded and was digested in the assays (Fig. 3B, white bar). Regardless of the heterogeneity detected by oligonucleotide *P1* hybridization, this finding indicated that almost all *trans*-δ-Rz forms (i.e. regardless of whether or not the P1 helix was folded or unfolded) have their L3 loop exposed. Folded *trans*-δ-Rz was detected only under native conditions in the presence of the cognate substrate. Under these conditions, approximately 30% of *trans*-δ-Rz was cleaved (Fig. 3B, dotted white bar). These findings suggest that the proper folding of the L3 loop in the complex between *trans*-δ-Rz and its cognate substrate requires the presence of the divalent metal ion, i.e. Mg^{2+}. This notion supports those of previous reports using chemical interference methodology (i.e., DMS or phosphate modification, Refs. 21, 22). In the analogous assays with *cis*-δ-Rz (1 nM), approximately 90% remained unfolded and was cleaved under partially denaturing conditions (Fig. 3B, dotted black bar); whereas 10% was cleaved or unfolded under native conditions (Fig. 3B, dotted white bar). The accessibility of the L3 loop of *cis*-δ-Rz was similar to that of *trans*-δ-Rz under both partially denaturing and native con-

ditions. The lack of RNase H cleavage of the L3 loop, referred to here as the folded L3 loop, can be attributed to the involvement of the nucleotides of the L3 loop in formation of the P1.1 stem. This stem was recently discovered by crystallography (11), and the base-pair interactions between the L3 loop and the J1/4 helix were reported to be crucial for the catalytic activity of δ ribozymes (12, 13). The finding that the L3 loop is 'folded' only in the presence of both the cognate substrate and the divalent ion, i.e., Mg^{2+}, is a clear indication that the P1.1 stem forms exclusively in the presence of the metal ion. Although the crystallographic data show no metal ion in the structure (11), the presence of metal ions in the ribozyme catalytic steps cannot be excluded. It is therefore logical to postulate that metal ions are transiently required for δ ribozyme cleavage, similar to that lead ions were required in the $tRNA^{phe}$ (23). In addition, our previous studies on metal induced cleavage of δ ribozyme show that formation of the P1 stem caused a specific cleavage of the J4/2 helix, strongly suggesting the requirement for Mg^{2+} (24). Taking into account the fact that divalent ions are absolutely required for the catalytic activity of δ ribozymes (13–14), it is very likely that either the proper folding of the L3 helix, or P1.1 stem formation, greatly contributes to the catalytic pathway, specifically to active site formation. Consequently, it is important to further characterize the kinetics of the L3 loop folding, especially under the conditions in which the accessibility of the oligonucleotide *L3* was modulated by the change from partially denaturing to native conditions.

Monitoring of the Folding Kinetics of δ Ribozymes Using Oligonucleotide L3 Hybridization and RNase H Cleavage Assays

The folding rate of the L3 loop as a function of the Mg^{2+} concentration was determined by measuring the level of oligonucleotide *L3* dependent RNase H cleavage following the incubation of ribozyme reaction mixtures in folding buffer containing the substrate and various Mg^{2+} concentrations (Fig. 4). In order to maintain the RNase H cleavage activity, the final Mg^{2+} concentration in the RNase H cleavage buffer was adjusted to 10 mM. The availability of Mg^{2+} significantly influenced the folding rate of *trans*-δ-Rz. Very little if any folding of the L3 loop was observed at 0.5 mM $MgCl_2$, as shown by the high level of *L3* dependent RNase H activity observed at all time points (Fig. 4A, clear boxes). Higher magnesium concentrations, such as 2 and 10 mM, significantly increased the folding rate (Fig. 4A, compare the filled and clear circles to the clear boxes).

Interestingly, a constant folding rate was observed at 2 mM magnesium, whereas a double exponential
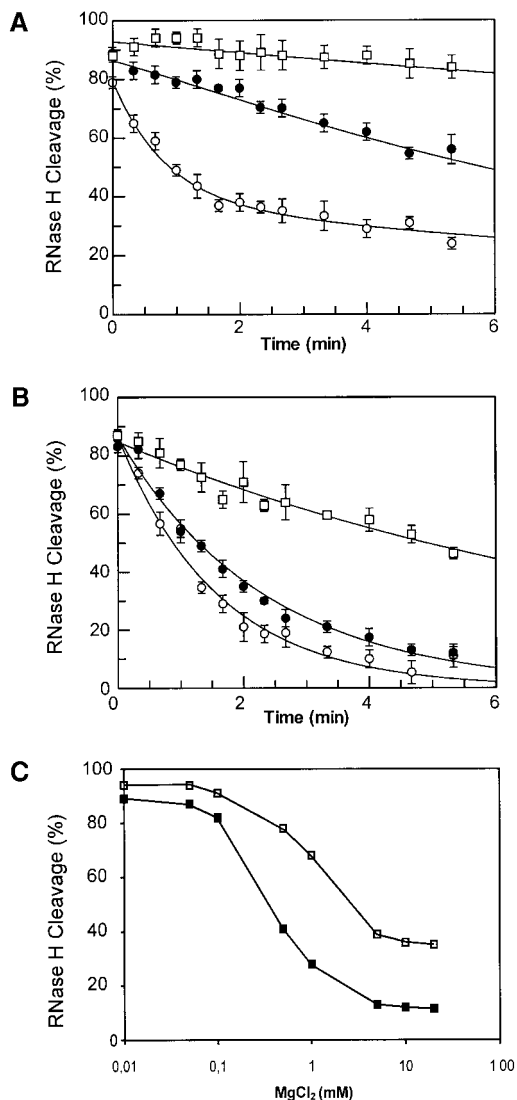
FIG. 4. Folding kinetics of the L3 loop. (A) Folding of *trans*-δ-Rz in the presence of its substrate. Time course plots of Mg^{2+} induced folding using probe *L3* and various concentrations of $MgCl_2$ (0.5 mM (□), 2 mM (●) and 10 mM (○). (B) Time course plots of self-cleaved *cis*-δ-Rz folding under conditions similar to those used for the self cleavage reaction. The rate of folding observed at 10 mM $MgCl_2$ represents what occurs in the enzymatic assays. (C) Equilibrium magnesium dependency of *trans*- and *cis*-δ-Rz. Each point represents the end point of folding kinetic experiments for *trans*-δ-Rz (■) and *cis*-δ-Rz (□) carried out at various concentrations of $MgCl_2$. Values of $[Mg]_{1/2}$, or K_{Mg}, were found to be 2–3 and 0.5–0.6 mM for *trans*-δ-Rz and *cis*-δ-Rz, respectively.

decay rate was obtained at 10 mM (0.23 ± 0.03 min^{-1} and 0.09 ± 0.01 min^{-1}, Fig. 4A). One possible explanation for this phenomenon is that after 2 min, equilibrium is reached between the levels of folded and unfolded derivatives of *trans*-δ-Rz. Alternatively, since the presence of substrate affects the folding of the L3 loop, the level of substrate or product remaining over time may influence the folding rate. To verify this

second postulate, the folding rate of the L3 loop was determined in reaction mixtures in which the cognate substrate was replaced by either a chemically modified uncleavable substrate (SdC4), or the product (RNA-P2). The rates of L3 loop inaccessibility were fitted to a single exponential equation, and calculated to be 0.12 ± 0.02 min^{-1} and 0.11 ± 0.05 min^{-1} with SdC4 and RNA-P2, respectively. These results suggest that product accumulation may interfere with folding. The uncleavable substrate may mimic the product effect, since it possesses in part the same sequence information and, as is the case with the product, cannot be cleaved.

cis-δ-Rz exhibited a lower magnesium requirement for proper folding of the L3 loop than did *trans*-δ-Rz (Fig. 4B). In contrast to *trans*-δ-Rz, a significant level of resistance to *L3* driven RNase H cleavage was observed magnesium concentrations as low as 0.5 mM (Fig. 4B, clear boxes), and the maximal exponential decay rate was observed to be significantly higher (0.61 ± 0.05 min^{-1}). The optimal mid-point magnesium concentration required for a maximal level of folding of the L3 loop was determined to be 2–3 mM for *trans*-δ-Rz and 0.5–0.6 mM for *cis*-δ-Rz (Fig. 4C). The magnesium requirement for the catalytic cleavage for *trans*-δ-Rz was previously reported to be 2.2 mM (18).

When the folding data are correlated with enzymatic activity, there are several interesting values worth mentioning. Although in previous studies the enzymatic reactions were performed in slightly different buffers (50 mM Tris pH 8.0, 10 mM $MgCl_2$) (18, 25), here the folding experiments are performed in the same buffer with the addition of 10 mM NaCl, 0.01 mM EDTA, and 1 mM dithiothreitol. Using the folding buffer for enzymatic assays, *cis*-δ-Rz exhibited an observed cleavage rate (k_{obs}) of 2.55 ± 0.01 min^{-1} and a $t_{1/2}$ of 0.27 min. Under single-turnover conditions, *trans*-δ-Rz had a $k_{obs} = 0.38 \pm 0.04$ min^{-1} and a $t_{1/2} = 1.8$ min. Their kinetic values are comparable to those reported previously (18, 25), consequently the folding behavior studied here reflects that in the enzymatic assay. As a result we took our previous kinetic studies into consideration in order to correlate folding with catalytic pathway as discussed below.

As shown in Fig. 5, *trans*-δ-Rz recognizes its cognate substrate, resulting in formation of the P1 stem. This substrate association requires only the presence of a monovalent ion, such as Na$^+$, as shown in this study (step 1). The folding and kinetic intermediate, whose L3 loop is folded, or participates in the formation of the P.1.1 stem, is formed in the presence of $MgCl_2$ (step 2). Under conditions permissive for both catalysis and folding (i.e., 10 mM $MgCl_2$), the rate at which the L3 loop becomes inaccessible to oligonucleotide *L3* driven RNase H cleavage is 0.23 ± 0.03 min^{-1}. We also used oligonucleotide *L3* as an inhibitor of *trans*-δ-Rz cleav-
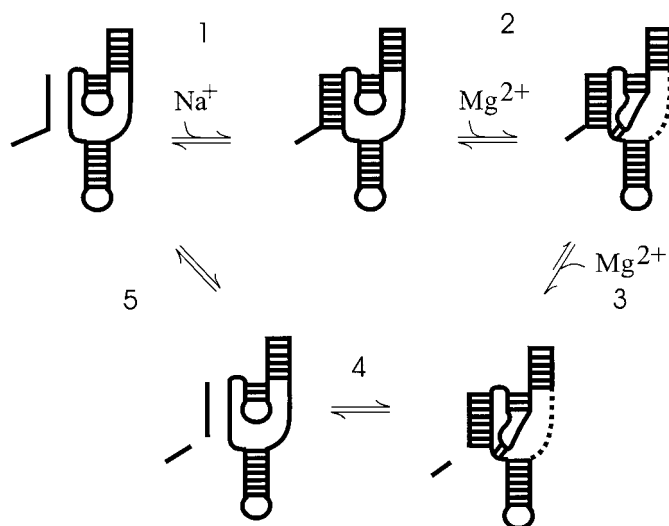
FIG. 5. Schematic representation of δ ribozyme folding and catalytic pathway.

age, and found that this oligonucleotide can decrease the rate of cleavage under both single- and multiple-turnover conditions (data not shown). Next, the phosphate backbone of the substrate is cleaved, and the first cleavage product is released (step 3). According to our previous studies under single-turnover conditions, the observed rate of cleavage (k_2) is 0.29 ± 0.03 min^{-1} (18, 25). It should be noted that this step might involve some nucleotides located on the J4/2 helix as described by Lafontaine *et al.* (24). In addition, a recent study by Perrotta *et al.* (19) showed that a nucleotide (cytosine) in the J4/2 helix requires the presence of imidazole buffer when this position was changed to adenosine. Consequently it suggests the catalytic function of the conserved nucleotide of J4/2. In the penultimate step the second cleavage product is released in a process which might involve unfolding of the L3 loop (step 4). This step has been shown to be rate limiting under multiple-turnover conditions (25). Finally, the *trans*-δ-Rz is recycled (step 5). Since the observed rate of L3 folding, and the k_2 value are within the same order of magnitude, the answer to the question as to which step is the overall rate limiting step seems to be either the folding of L3 or the chemical cleavage. This finding is a major step forward in our understanding of the formation of the intermediates and tertiary structures involved in the δ ribozyme catalytic pathway.

In summary, we describe the folding of delta ribozymes using an oligonucleotide hybridization assay which has previously been used for large ribozymes including the group I intron and RNase P (9, 10). This is the first demonstration that a small ribozyme (i.e., δ ribozyme) contains some helices and loops that are sensitive (P1, L3, and J4/2), and others that are insensitive (J1/4, P4) to the presence of divalent metal ions

in a manner similar to that found in the large ribozymes (9, 10). We clearly show a specific function of Mg^{2+} in supporting either the formation of the P1.1 stem, or in the L3 loop folding.

REFERENCES

1. Narlikar, G. J., and Herschlag, D. (1997) Mechanistic aspects of enzymatic catalysis: Lessons from comparison of RNA and protein enzymes. *Annu. Rev. Biochem.* **66,** 19–59.

2. Turner, P. C. (1997) Methods in Molecular Biology: Ribozyme Protocols, Vol. 74, Humana Press, Totowa, NJ.

3. Pley, H. W., Flaherty, K. M., and McKay, D. B. (1994) Model for an RNA tertiary interaction from the structure of an intermolecular complex between a GAAA tetraloop and an RNA helix. *Nature* **372,** 111–113.

4. Pley, H. W., Lindes, D. S., DeLuca-Flaherty, C., and McKay, D. B. (1993) Crystals of a hammerhead ribozyme. *J. Biol. Chem.* **268,** 19656–19658.

5. Scott, W. G., Finch, J. T., and Klug, A. (1995) The crystal structure of an all-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell* **81,** 991–1002.

6. Scott, W. G., Murray, J. B., Arnold, J. R. P., Stoddard, B. L., and Klug, A. (1996) Capturing the structure of a catalytic RNA intermediate: The hammerhead ribozyme. *Science* **274,** 2065–2069.

7. Walter, F., Murchie, A. I. H., and Lilley, D. M. J. (1998) Folding of the four-way RNA junction of the hairpin ribozyme. *Biochemistry* **37,** 17629–17636.

8. Bassi, G. S., Murchie, A. I. H., Walter, F., Clegg, R. M., and Lilley, D. M. J. (1997) Ion-induced folding of the hammerhead ribozyme: A fluorescence resonance energy transfer study. *EMBO J.* **16,** 7481–7489.

9. Zarrinkar, P. P., and Williamson, J. R. (1994) Kinetic intermediates in RNA folding. *Science* **265,** 918–924.

10. Zarrinkar, P. P., Wang J., and Williamson, J. R. (1996) Slow folding kinetics of RNase P RNA. *RNA* **2,** 564–573.

11. Ferre-D'Amare, A. R., Zhou, K., and Doudna J. A. (1998) Crystal structure of a hepatitis delta virus ribozyme. *Nature* **395,** 567–574.

12. Wadkins, T. S., Perrotta, A. T., Ferre-D'Amare, A. R., Doudna, J. A., and Been, M. D. (1999) A nested double pseudoknot is required for self-cleavage activity of both the genomic and antigenomic hepatitis delta virus ribozymes. *RNA* **5,** 720–727.

13. Nishikawa, F., and Nishikawa, S. (2000) Requirement for canonical base pairing in the short pseudoknot structure of genomic hepatitis delta virus ribozyme. *Nucleic Acids Res.* **28,** 925–931.

14. Murray, J. B., Seyhan, A. A., Walter, N. G., Burke, J. M., and Scott, W. G. (1998) The hammerhead, hairpin and VS ribozymes are catalytically proficient in monovalent cations alone. *Chem. Biol.* **5,** 587–595.

15. Wu, H.-Y., and Lai, M. M. (1990) RNA conformational requirements of self-cleavage of hepatitis delta virus RNA. *Mol. Cell. Biol.* **10,** 5575–5579.

16. Gottlieb, P. A., Prasad, Y., Smith, J. B., Williams, A. P., and Dinter-Gottlieb, G. (1994) Evidence that alternate foldings of the hepatitis delta RNA confer varying rates of self-cleavage. *Biochemistry* **33,** 2802–2808.

17. Wu, H.-N., Lee, J.-Y., Ping, Y.-H., and Wang, C.-N. (1998) The effect of Lp3 enlargement on the folding and catalysis of hepatitis delta virus *cis*-cleaving ribozyme. *FEBS Lett.* **439,** 312–316.

18. Ananvoranich, S., and Perreault, J. P. (1998) Substrate specificity of delta ribozyme cleavage. *J. Biol. Chem.* **273,** 13182–13188.

19. Perrotta, A. T., Shih, I., and Been M. D. (1999) Imidazole rescue of a cytosine mutation in a self-cleaving ribozyme. *Science* **286,** 123–126.

20. Fauzi, H., Kawakami, J., Nishikawa, F., and Nishikawa S. (1997) Analysis of the cleavage reaction of a *trans*-acting human hepatitis delta virus ribozyme. *Nucleic Acids Res.* **25,** 3124–3130.

21. Puglisi, J. D., Wyatt, J. R., and Tinoco, I., Jr. (1990) Conformation of an RNA pseudoknot. *J. Mol. Biol.* **214,** 437–453.

22. Jeoung, Y. H., Kumar, P. K., Suh, Y. A., Taira, K., and Nishikawa, S. (1994) Identification of phosphate oxygens that are important for self-cleavage activity of the HDV ribozyme by phosphorothioate substitution interference analysis. *Nucleic Acids Res.* **22,** 3722–3727.

23. Pan, T., Long, D. M., and Uhlenbeck, O. C. (1993) *In* The RNA World (Gesteland, R. F., and Atkins, J. F., Eds.), pp. 371–302. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

24. Lafontaine, D. A., Ananvoranich, S., and Perreault, J. P. (1999) Presence of a coordinated metal ion in a *trans*-acting antigenomic delta ribozyme *Nucleic Acids Res.* **27,** 3236–3243.

25. Mercure, S., Lafontaine, D., Ananvoranich, S., and Perreault, J. P. (1998) Kinetic analysis of delta ribozyme cleavage. *Biochemistry* **37,** 16975–16982.

26. Been, M. D., Perrotta, A. T., and Rosenstein, S. P. (1992) Secondary structure of the self-cleaving RNA of hepatitis delta virus: Applications to catalytic RNA design. *Biochemistry* **31,** 11843–11852.