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The influence of junction conformation on RNA cleavage by the hairpin ribozyme in its natural junction form

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ABSTRACT

In the natural form of the hairpin ribozyme the two loop-carrying duplexes that comprise the majority of essential bases for activity form two adjacent helical arms of a four-way RNA junction. In the present work we have manipulated the sequence around the junction in a way known to perturb the global folding properties. We find that replacement of the junction by a different sequence that has the same conformational properties as the natural sequence gives closely similar reaction rate and Arrhenius activation energy for the substrate cleavage reaction. By comparison, rotation of the natural sequence in order to alter the three-dimensional folding of the ribozyme leads to a tenfold reduction in the kinetics of cleavage. Replacement with the U1 four-way junction that is resistant to rotation into the antiparallel structure required to allow interaction between the loops also gives a tenfold reduction in cleavage rate. The results indicate that the conformation of the junction has a major influence on the catalytic activity of the ribozyme. The results are all consistent with a role for the junction in the provision of a framework by which the loops are presented for interaction in order to create the active form of the ribozyme.

Keywords: four-way junction; metal ions; RNA catalysis

INTRODUCTION

The small nucleolytic ribozymes (reviewed in Eckstein & Lilley, 1996) provide an excellent opportunity to study the relationship between RNA structure and activity. One well studied example is the hairpin ribozyme, which is the self-processing motif of the negative strand of the satellite RNA of the tobacco ringspot virus (Buzayan et al., 1986; Feldstein et al., 1989; Hampel & Tritz, 1989) and similar plant viruses (DeYoung et al., 1995). Following replication by a rolling circle mechanism, the concatameric RNA becomes processed into monomeric RNA molecules by RNA-mediated self cleavage due to the activity of the hairpin ribozyme. The ribozyme also carries out the reverse reaction (Buzayan et al., 1986), leading to circularization of the monomeric RNA (van Tol et al., 1991), and thus to an equilibrium between cleavage and ligation (Hegg & Fedor, 1995).

The critical elements required for the activity of the hairpin ribozyme are two short duplex sections each interrupted by formally unpaired loops (generally called the A and B loops), that are connected by virtue of containing a common strand. In the secondary structure of the viral RNA, these two duplexes are adjacent arms of a perfect four-way helical junction (4H junction; Lilley et al., 1995). The activity of the ribozyme is preserved if the two additional arms of the junction are removed (Hampel & Tritz, 1989; Hampel et al., 1990; Chowrira et al., 1991), generating a form where the two duplexes may hinge about the common strand, and the majority of studies of this ribozyme have concentrated on this form. It was suspected that physical contact was required between the A and B loops in order to generate the active ribozyme. Thus the majority of essential bases (Berzal-Herranz et al., 1993; Anderson et al., 1994; Siwkowski et al., 1997) and functional groups (Chowrira et al., 1993; Grasby et al., 1995; Schmidt et al., 1996) are located in these loops. Furthermore, activity was found to be retained when the loop-carrying arms were connected together in a variety of ways (Komatsu et al., 1994, 1996, 1997a, 1997b; Butcher et al., 1995; Earnshaw et al., 1997), and some activity was even observed *in trans* with unconnected loop-carrying duplexes (Butcher et al., 1995; Shin et al., 1996). Interaction between the loops was sup-

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ported by chemical crosslinking studies (Earnshaw et al., 1997), and very recently we and others have provided direct physical evidence for the close approach of the loop-bearing duplexes using fluorescence resonance energy transfer (FRET) (Murchie et al., 1998; Walter et al., 1998c).

While the RNA around the hinge point of the minimized form of the hairpin ribozyme should provide a point of relatively simple flexibility, the conformation of the natural form of the ribozyme is likely to be rather more complicated. In the viral RNA the four-way junction will be stereochemically very much more constrained than a flexible hinge, and the conformation of the junction would be expected to provide a strong influence on the ability of the loops to make contact, and hence on the activity of the ribozyme. RNA four-way junctions are know to have precise global structures (Duckett et al., 1995; Walter et al., 1998a). Using FRET we have shown that the helical arms of the junction form of the hairpin ribozyme are pairwise coaxially stacked with A on D and B on C stacking partners. Moreover, in the presence of magnesium ions, the junction rotates into a distorted antiparallel global conformation that brings the A and B arms into physical proximity, consistent with a close association between the A and B loops (Murchie et al., 1998; Walter et al., 1998b). The junction provides a framework on which the loops are presented for association, and it is likely to play an important structural role in the formation of the active ribozyme. In this article we have tested this concept by modifying the sequence of the junction in a way that should perturb the overall conformation of the molecule in a predictable way. We find that the cleavage activity of the ribozyme can be significantly modulated by the stereochemistry of the four-way junction, in a manner consistent with a requirement for the correct presentation of the loops.

RESULTS

Dependence of the rate of cleavage by the hairpin ribozyme on the conformation of the four-way junction

We have shown previously that the hairpin ribozyme is active in its natural four-way junction context, and that forcing the conformation of the junction into the alternative stacking conformer significantly reduces the activity of the ribozyme (Murchie et al., 1998). This raises the question of how important the sequence of the junction is, as opposed to its conformation. We therefore replaced the natural sequence of the junction (the innermost 2 bp of each arm) by that of a different four-way junction. The global conformation of junction 3 is well understood from earlier studies, and the stacking preference has been established by comparative gel electrophoresis (Duckett et al., 1995). The junction 3 sequence was inserted into the ribozyme with an orientation chosen to place the A and B loops on opposite helical stacks (i.e., A on D and B on C stacking) as in the natural ribozyme (Fig. 1).

The kinetics of cleavage of the modified ribozyme containing the junction 3 sequence were compared with the natural sequence hairpin ribozyme. Cleavage rates were studied using a two-stranded species in which a transcribed ribozyme strand was hybridized to a [5′-^{32}P]-labeled synthetic substrate strand. The reaction was carried out in the presence of 10 mM magnesium ions under single-turnover conditions at 25 °C, and the products analyzed by gel electrophoresis. The extent of product formation was quantified by phosphorimaging, and plotted in Figure 2. It can be seen that the rates of cleavage of the natural ribozyme and that em-

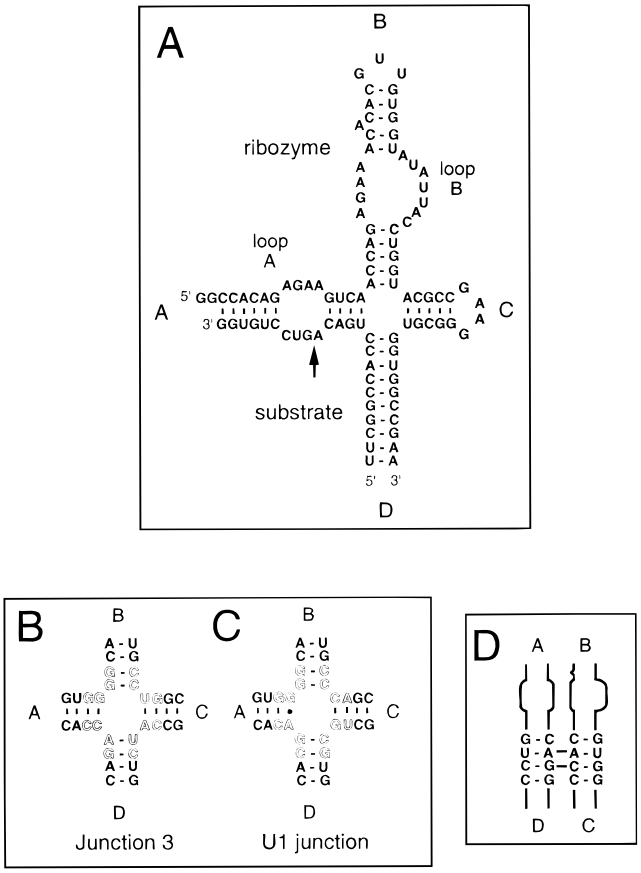

FIGURE 1. The sequence of the hairpin ribozyme. **A**: The natural sequence of the ribozyme in the two-stranded form used for the study of cleavage kinetics. The position of cleavage is indicated by the arrow. **B**: The central sequence of junction 3 (Duckett et al., 1995), shown in the same orientation as that used in the ribozyme shown in **A**. **C**: The central sequence of the U1 junction, shown in the same orientation as that used in the ribozyme. This junction contains an A•G mismatch at the point of strand exchange, but this feature does not interfere with coaxial stacking of arms (Duckett et al., 1995; Walter et al., 1998a). **D**: The stacking conformer adopted by all three junctions in the orientation used. The junctions preferred choice of stacking partners is A arm on D and B on C, and thus the A and B loops are on different stacked pairs as shown, where they can potentially interact. The sequence shown is that of the natural ribozyme.

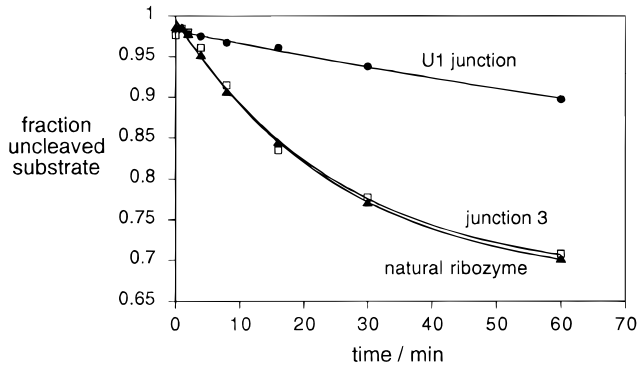

FIGURE 2. The progress of the cleavage reaction by the hairpin ribozyme in its natural form, and with the replaced junction sequences. Preincubated ribozyme (1 μM) and substrate (50 nM) were incubated in 50 mM Tris-HCl (pH 7.5) at 25 °C in the presence of 10 mM magnesium ions. Aliquots were removed at increasing times, and product formation quantified by polyacrylamide gel electrophoresis and phosphorimaging. The data are plotted as the fraction of uncleaved/total substrate as a function of time, and fitted to a single exponential. ▲, natural ribozyme; □, junction 3-based ribozyme; ●, U1 junction-based ribozyme.

ploying the sequence of junction 3 are closely similar, with $k_{obs} = 0.038 \pm 0.006$ min^{-1} for both ribozymes under these conditions. Since the base sequences of the junctions are quite different, we conclude that the conformation of the four-way junction, rather than its sequence, determines the rate of cleavage.

We have measured the rates of substrate cleavage of the natural hairpin ribozyme in the presence of 10 mM calcium and strontium ions, using the two-stranded construct under single-turnover conditions at 25 °C as before (data not shown). The cleavage rate in the presence of strontium ions corresponds to $k_{obs} = 0.024 \pm 0.004$ min^{-1}, whereas the rate in the presence of calcium ions is significantly faster with $k_{obs} = 0.09 \pm 0.01$ min^{-1}. The difference in rates between the ions is highly reproducible.

Effect of the U1 junction on ribozyme cleavage—a junction hindered in rotation to the antiparallel form

To explore the role of junction conformation further we employed a third junction sequence, that of the U1 snRNA (Branlant et al., 1981). Although this junction undergoes pairwise coaxial stacking into one preferred conformer, it differs from other four-way RNA junctions studied in that it is reluctant to rotate into the antiparallel conformation even at high magnesium concentrations (Duckett et al., 1995; Walter et al., 1998a). If this remains true when the sequence is transplanted into the hairpin ribozyme, this should hinder the association between the loops and thus reduce the efficiency of ribozyme cleavage. We therefore made an analogous two-stranded form of the ribozyme-substrate complex replacing the junction with the sequence from U1 snRNA, choosing the orientation to preserve the A on D, B on C stacking (see Fig. 1). Using this ribozyme it can be seen that the rates of substrate cleavage (Fig. 2) are significantly slower that the natural or junction 3-based constructs, with $k_{obs} = 0.005 \pm 0.001$ min^{-1}. Evidently the conformation of the junction has affected the activity of the ribozyme.

Dependence of the rate of cleavage by the junction form of the hairpin ribozyme on magnesium ion concentration

The rates of substrate cleavage for the natural hairpin ribozyme and that based on junction 3 have been studied as a function of magnesium ion concentration (Fig. 3). Reactions were performed using the two-stranded constructs under single-turnover conditions at 25 °C. Both species require magnesium ion concentrations in the millimolar range for maximal activity. However, the natural sequence can be activated to higher k_{obs} values at elevated magnesium ion concentrations. The shape of the curves suggests that the rates are dependent on noncooperative binding by magnesium, and the data can be well fitted by a model that assumes the binding of a single magnesium ion is required to activate the ribozymes. This has been explored for the junction 3-based ribozyme in an alternative format, using a Hill plot. The data conform to a straight line, giving a Hill coefficient of 1.09 ± 0.03. This again indicates that the rate of cleavage is affected by the noncooperative binding of a single magnesium ion. The range of magnesium ion concentration affecting cleavage rate is far above that at which the change in global structure is observed by FRET (typically 0–100 μM magnesium ions; Murchie et al., 1998; Walter et al., 1998b).

Temperature dependence of cleavage rates by the junction form of the hairpin ribozyme

We have studied the rate of cleavage of the natural hairpin ribozyme and that based on junction 3 in the presence of 10 mM magnesium ions as a function of temperature. This was carried out using the same two-stranded constructs under single-turnover conditions. The data are presented as Arrhenius plots in Figure 4. From the gradients of these plots we have calculated Arrhenius activation energies of 13.52 ± 1.91 and 13.50 ± 1.13 kcal mol^{-1} for the the natural and the junction 3-based ribozymes respectively. The close similarity in the activation energies for the two ribozymes indicates that the sequence of the junction has little effect on the temperature dependence of the cleavage reaction.

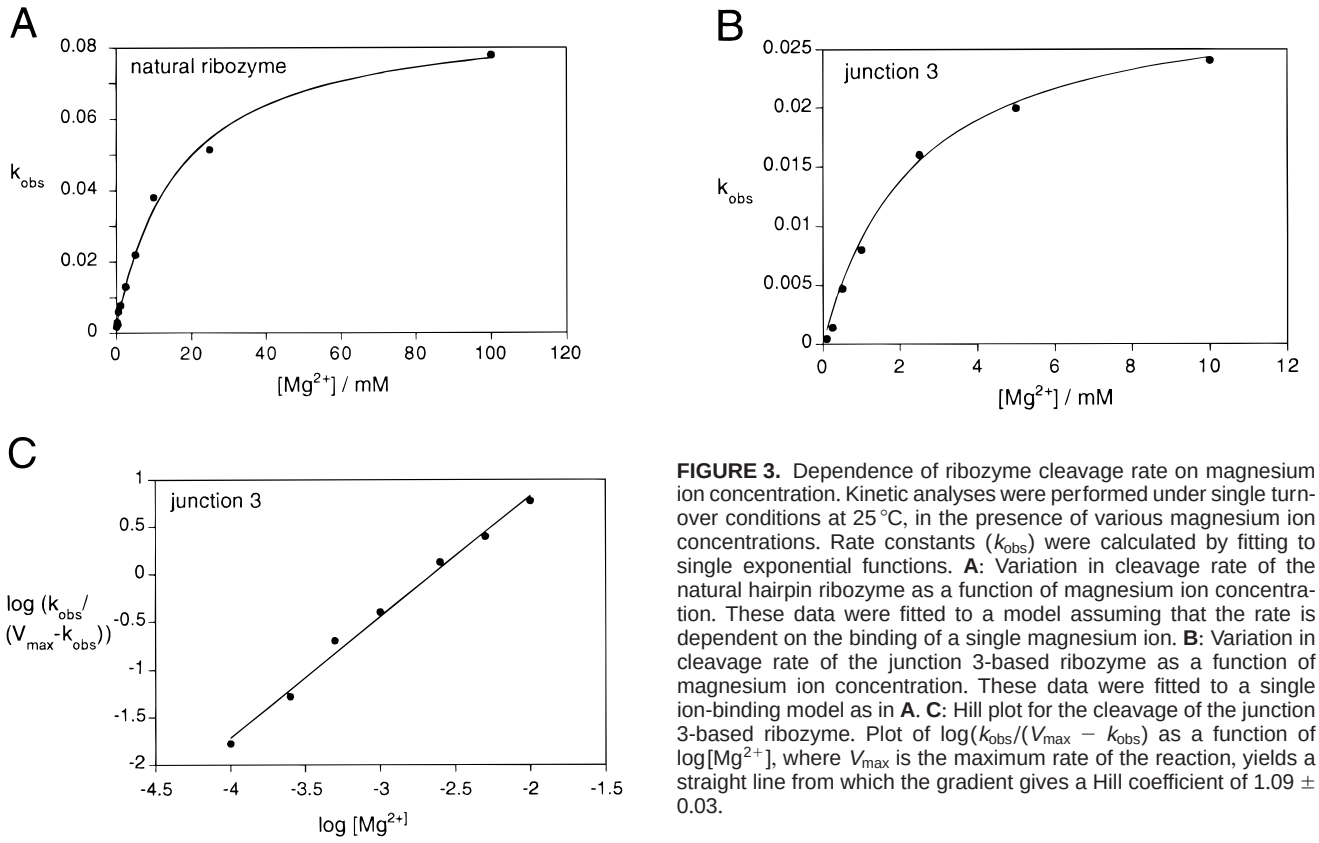

FIGURE 3. Dependence of ribozyme cleavage rate on magnesium ion concentration. Kinetic analyses were performed under single turnover conditions at 25 °C, in the presence of various magnesium ion concentrations. Rate constants (k_{obs}) were calculated by fitting to single exponential functions. **A**: Variation in cleavage rate of the natural hairpin ribozyme as a function of magnesium ion concentration. These data were fitted to a model assuming that the rate is dependent on the binding of a single magnesium ion. **B**: Variation in cleavage rate of the junction 3-based ribozyme as a function of magnesium ion concentration. These data were fitted to a single ion-binding model as in **A**. **C**: Hill plot for the cleavage of the junction 3-based ribozyme. Plot of $\log(k_{obs}/(V_{max} - k_{obs})$ as a function of $\log[Mg^{2+}]$, where V_{max} is the maximum rate of the reaction, yields a straight line from which the gradient gives a Hill coefficient of 1.09 ± 0.03.

DISCUSSION

Previous studies have provided a clear indication that the two loops of the hairpin ribozyme must come into association to generate the active conformation (Komatsu et al., 1994, 1996, 1997a, 1997b; Butcher et al., 1995; Shin et al., 1996). This close association of the A and B arms has recently been confirmed physically by FRET experiments (Murchie et al., 1998; Walter et al., 1998c), and is also consistent with chemical crosslink-

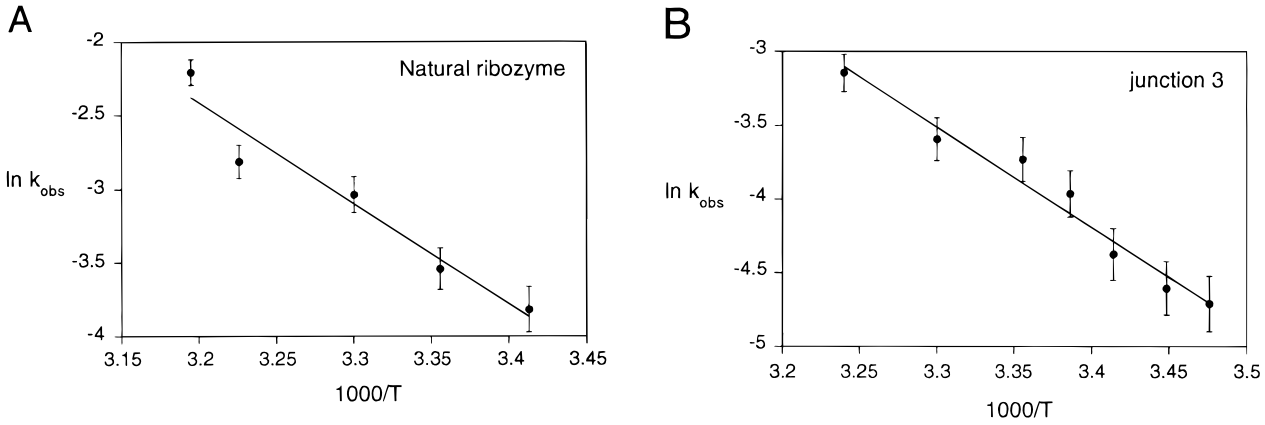

FIGURE 4. Temperature dependence of the junction forms of the hairpin ribozyme. Substrate cleavage by the natural and junction 3-based forms of the hairpin ribozyme were performed as a function of temperature in the presence of 10 mM magnesium ions under single-turnover conditions. The data were fitted to single exponentials, and rate constants calculated. These have been presented in the forms of Arrhenius plots. **A**: Temperature dependence of the cleavage reaction catalyzed by the natural sequence ribozyme. **B**: Temperature dependence of the cleavage reaction catalyzed by the junction 3-based ribozyme. The gradients of both sets of data are closely similar, corresponding to equal Arrhenius activation energies within experimental error.

ing studies (Earnshaw et al., 1997). In the natural ribozyme these arms are adjacent helices of a four-way junction, and it is expected that the junction will provide a framework on which the ribozyme is constructed. In this work we have analyzed the influence of the structure of the four-way junction on RNA-mediated ribozyme cleavage. In each case we have found that the substrate is cleaved at the same phosphodiester bond, but the rate of cleavage may vary. Our results have shown that the conformation of the four-way junction, but not its sequence, significantly affects the RNA catalyzed substrate cleavage rate. This has been shown by the following results (illustrated in Fig. 5):

1. Replacing the natural junction of the ribozyme with that of junction 3 changes the sequence of the junction but should preserve the same global structure (with A on D and B on C stacking). Loop-loop interaction is possible in this conformer, and the result is a hybrid ribozyme with very similar kinetic properties to the natural sequence. The main difference between these ribozymes lies in the magnesium dependence of the reaction rate.
2. Rotation of the natural sequence about the center of the junction preserves the sequence but should lead to a tendency to adopt the alternative stacking conformer. In this form the two loops are now located helices that are coaxially stacked, and thus loop-loop interaction would require disruption of this structure in some manner. The result is a tenfold reduction in the rate of cleavage (Murchie et al., 1998).
3. Replacement of the junction sequence by that of the U1 snRNA junction is likely to preserve the correct stacking conformer, but should nevertheless provide an impediment to loop-loop interaction of a different kind. The U1 junction has a marked tendency to remain in the 90° crossed structure, presenting a hindrance to rotation into the antiparallel structure (Duckett et al., 1995; Walter et al., 1998a; Fig. 5B). We find that this change also leads to a tenfold reduction in cleavage rate.

Thus provided the junction has the same conformational properties, ribozyme cleavage is largely unaffected, despite changes in sequence. However, alteration of the conformation, either by changing the choice of stacking partner (thus placing the loops onto the same pair of stacked helices) or interfering with the rotation that brings the loops together, leads to reduced activity. The resulting activity of the conformationally impaired ribozymes remains significant however; this could be an indication that the loop-loop interaction is sufficiently strong to partially overcome the conformational preferences of the junction, perhaps pulling the stacking equilibrium towards the A on D conformer in the case of the rotated junction, and forcing the rotation in the case of the U1 junc-

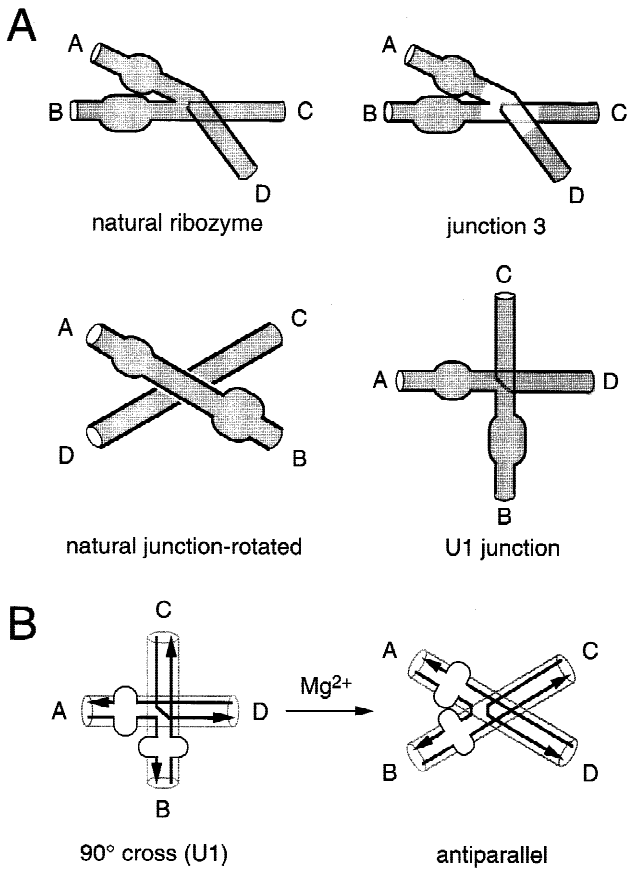

FIGURE 5. Schematic illustrating expected folding of the junction-variant hairpin ribozyme forms. **A**: The folding of the natural sequence ribozyme has been deduced from FRET experiments (Murchie et al., 1998; Walter et al., 1998b), illustrated by the schematic at top left. The junction stacks arms A on D and arms B on C, and the shortest end-to-end vector is the A–B distance. Junction 3 (top right) has a different sequence from the natural ribozyme sequence, but this junction has been introduced into the ribozyme so as to preserve the A on D, B on C stacking and thus permit the interaction between the A and B loops. Despite the change in junction sequence, this ribozyme is unaltered in activity from the natural sequence. The rotated version of the natural ribozyme (bottom left) has the same junction sequence (though permuted around the point of strand exchange by 90°) as the natural hairpin ribozyme. However the rearranged junction places the A and B loops onto the same helical axis. Rotation of this conformer cannot possibly bring the loops close together, and this form of the ribozyme has lowered cleavage activity (Murchie et al., 1998). The sequence of the U1 junction was placed into the hairpin ribozyme so as to preserve the stacking conformer that places the A and B loops onto different helical axes. However, this junction is very resistant to rotation into the antiparallel form, and tends to remain as a 90° crossed structure (bottom right, see also **B**). This form of the ribozyme has lowered cleavage activity, consistent with the difficulty of achieving loop-loop interaction if rotation to the antiparallel junction is hindered. **B**: Rotation of the junction from the 90° crossed structure that exists in the presence of low magnesium ion concentrations to the antiparallel structure. This transition is promoted by divalent metal ions such as magnesium, but does not occur readily for the U1 junction (Duckett et al., 1995; Walter et al., 1998a). Rotation into the antiparallel form is required to bring the loops on the A and B arms into close proximity, and is therefore likely to be required for catalytic activity. It is likely that a strong association between the loops could act to pull the U1 junction-based ribozyme into an antiparallel form.

tion. A strong interaction between the loops is also indicated by the asymmetrical global structure of the ribozyme (Murchie et al., 1998). It is interesting to note that the sequences that were varied in these studies (the innermost two base pair of each arm at the junction) can be changed without altering ribozyme activity in the hinged form of the ribozyme (i.e., the ribozyme lacking the C and D arms; Berzal-Herranz et al., 1993). In this case there is no junction present, just a flexible hinge. Thus it would not be expected that these sequences would affect the activity of the ribozyme in this case.

We have found that the activity of the ribozyme in its junction form is affected by magnesium ion concentration in the millimolar range. The kinetic data suggest that the cleavage rate is sensitive to the noncooperative binding of a single ion. This appears to be distinct from the ion-induced folding detected by FRET (Murchie et al., 1998), where we observe cooperative ion binding in the micromolar range. We conclude that the FRET and the cleavage experiments are therefore detecting the consequences of different binding events. Recent demonstrations of cleavage activity in the presence of the substitutionally inert hexammine cobalt (III) complex (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997) appear to exclude a direct role for the metal ion as either a Lewis acid or in general base catalysis, and thus the effect of the lower affinity ion on cleavage rate is likely to be structural. Since no change in FRET efficiencies are detected in the millimolar range of magnesium ion concentration, any structural effects must be local such that the global shape of the ribozyme is essentially unaffected. It therefore seems probable that an additional metal ion is coordinated into the loop interface in order to generate the local stereochemistry required in the transesterification reaction. The difference in magnesium ion concentration range required to achieve the activation between the natural sequence and junction 3 indicates that some aspect of the junction conformation must be different, and that this facilitates the metal ion coordination into the junction 3 construct. Our earlier finding that while manganese ions apparently fold the hairpin ribozyme normally while not leading to detectable cleavage reaction (Walter et al., 1998b) suggests that while the transition metal ion behaves similarly to magnesium in terms of the overall folding, it is unable to participate in this local manner.

The results obtained in this study show that the conformation of the four-way junction can exert a significant influence on the activity of the natural form of the hairpin ribozyme. Taken together the results are fully consistent with the junction acting as an important structural element in allowing the interaction between the loops. It is the framework that facilitates the formation of the tertiary structure required to create the catalytically competent conformation.

MATERIALS AND METHODS

Preparation of hairpin ribozyme and derived constructs

RNA was synthesized using phosphoramidite chemistry (Beaucage & Caruthers, 1981), using ribonucleotide phosphoramidites with 2′-*tert* butyldimethylsilyl (TBDMS) protection (Hakimelahi et al., 1981; Perreault et al., 1990; Glen Research). RNA was deprotected in 25% ethanol/ammonia solution at 55 °C for 12 h and evaporated to dryness. Oligoribonucleotides were redissolved in 0.5 mL 1 M tetrabutylammonium fluoride (Aldrich) in tetrahydrofuran to remove TBDMS groups, and agitated at 20 °C in the dark for 16 h prior to desalting by G25 Sephadex (NAP columns; Pharmacia) and ethanol precipitation. Fully deprotected oligonucleotides were purified by gel electrophoresis in 20% polyacrylamide containing 7 M urea, and electroeluted into 8 M ammonium acetate and recovered by ethanol precipitation. RNA was radioactively [5′-^{32}P] labelled using [γ-^{32}P]-ATP and polynucleotide kinase (Maxam & Gilbert, 1980).

Transcription of RNA

RNA was transcribed from synthetic DNA templates using T7 RNA polymerase according to Milligan et al. (1987), and purified by polyacrylamide gel electrophoresis.

Analysis of ribozyme cleavage

Cleavage experiments were carried out under single-turnover conditions (Thomson et al., 1996). Ribozyme and [5′-^{32}P]-labeled substrate were individually incubated in 50 mM Tris-HCl (pH 7.5) to 90 °C for 2 min, followed by 15 min at the indicated temperature. Metal salts were added to the required concentration, and incubation continued for a further 60 min. The reaction was initiated by mixing ribozyme and substrate at 1 μM and 50 nM final concentrations respectively. The cleavage reactions were stopped at the indicated times, and product formation was analyzed by electrophoresis on sequencing gels containing 7 M urea. Product formation was quantified by exposure to storage phosphor screens and phosphorimaging (BAS-1500, Fuji).

Progress curves (fraction uncleaved substrate (f) as a function of time) were fitted to a single exponential function,

$$f = e^{-k_{obs}t}$$

where t is the time of incubation and k_{obs} the observed rate constant. Repetitive measurement of rates indicate an experimental error of $\pm$15%. The magnesium ion concentration dependence of cleavage rate for the junction 3-based ribozyme was presented as a Hill plot, plotting $\log(k_{obs}/(V_{max} - k_{obs}))$ versus $\log[Mg^{2+}]$ ($[Mg^{2+}]$ in M). This was fitted to a straight line, and the Hill coefficient was obtained from the gradient.

The temperature dependence of cleavage reaction rate was presented as an Arrhenius plot, that is, fitting to the equation

$$k_{obs} = Ae^{-Ea/RT}$$

where *Ea* is the Arrhenius activation energy, *R* is the gas constant (1.987 cal deg^{-1} mol^{-1}), *T* the temperature (K) and *A* the preexponential factor. The error is the standard error on the linear fit.

For each construct the substrate strand was chemically synthesized whereas the ribozyme was prepared by transcription. All sequences are written 5′ to 3′:

Natural ribozyme
substrate: UUCGGCCA**CCUG**ACAGUCCUGUGG
ribozyme: GGCCACAGAGAAGU**CAAC**CAGAGAAACACAC
GUUGUGGUAUAUUACCUG**GUAC**GCCGAAAGGC**GUG**
GUGGCCGAA
Junction 3-based ribozyme
substrate: UUCGGCCA**GUCC**ACAGUCCUGUGG
ribozyme: GGCCACAGAGAAGU**GGGG**CAGAGAAACACA
CGUUGUGGUAUAUUACCUG**CCUG**GCCGAAAGGC**CA**
ACUGGCCGAA
U1 RNA-based ribozyme
substrate: UUCGGCCA**CGAC**ACAGUCCUGUGG
ribozyme: GGCCACAGAGAAGU**GGGG**CAGAGAAACACA
CGUUGUGGUAUAUUACCUG**CCCA**GCCGAAAGGC**UG**
CGUGGCCGAA

The sequences around the junctions that have been altered in these experiments are highlighted in bold.

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