Metal ion binding and the folding of the hairpin ribozyme

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ABSTRACT
The hairpin ribozyme comprises two formally unpaired loops carried on two arms of a four-way helical RNA junction. Addition of divalent metal ions brings about a conformational transition into an antiparallel structure in which there is an intimate association between the loops to generate the active form of the ribozyme. In this study, we have used fluorescence resonance energy transfer to analyze the global folding of the complete ribozyme, and the simple four-way junction derived from it, over a wide concentration range of divalent and monovalent metal ions. The simple junction undergoes an ion-induced rotation into an antiparallel form. In the presence of a constant background concentration of sodium ions, the magnesium-ion-induced transition is characterized by noncooperative binding with a Hill coefficient \( n = 1 \). By contrast, the magnesium-ion-induced folding of the complete ribozyme is more complex, involving two distinct binding phases. The first phase occurs in the micromolar range, and involves the cooperative binding of at least three magnesium ions. This can also be achieved by high concentrations of sodium ions, and is therefore likely to be due to diffuse binding of cations at the junction and the interface of the loop–loop interaction. The second phase occurs in the millimolar range, and can only be induced by divalent metal ions. This transition occurs in response to the noncooperative, site-specific binding of magnesium ions. We observe a good correlation between the extent of ion-induced folding and cleavage activity.

Keywords: FRET; RNA catalysis; RNA folding

INTRODUCTION
The hairpin ribozyme (Fedor, 2000) is a self-processing element that exists in 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). It is one of the small nucleolytic ribozymes that bring about the RNA-catalyzed cleavage of the backbone at a specific site in the molecule. This is achieved by a transesterification reaction, in which the neighboring 2'-oxygen attacks the 3'-phosphorus, with the departure of the 5'-oxygen. In the context of the ribozyme, this reaction is accelerated by a factor of around 10^5. The reverse ligation reaction is also efficiently catalyzed by the hairpin ribozyme.

Ribozyme activity requires precise folding of the RNA to generate the environment in which catalysis can occur. The hairpin ribozyme is constructed from two loop-bearing duplexes that exist as adjacent arms of a four-way junction (Fig. 1). The majority of essential bases (Berzal-Herranz et al., 1993; Anderson et al., 1994; Siwickowski et al., 1997) and functional groups (Chowrir et al., 1993; Grasby et al., 1995; Schmidt et al., 1996) are located in these loops, and it was supposed that close interaction between the loops would be required to generate the active form of the ribozyme, which was supported by chemical crosslinking studies (Earnshaw et al., 1997). Physical evidence for the interaction was obtained using fluorescence resonance energy transfer (FRET), proving the close approach of the ends of the two loop-carrying duplex arms (Murchie et al., 1998b, 1998c). Recently, the structure of the ribozyme in its junction form has been solved by X-ray crystallography (Rupert & Ferré-D’Amaré, 2001). This reveals the molecular detail of the close association between the loops, and provides insight into how the cleavage and ligation reactions may be accelerated.

Electrostatic interactions are extremely important in the conformational transitions of nucleic acids, due to their polyelectrolyte character. For this reason, metal ions generally play a critical role in mediating structural alterations. We have shown that in the absence of divalent metal ions, the hairpin ribozyme forms a struc-
ture in which the helical arms around the four-way junction are coaxially stacked in pairs (A on D, B on C) (Murchie et al., 1998), as is also observed in the crystal structure (Rupert & Ferré-D’Amaré, 2001), but that the axes are approximately perpendicular under these conditions (Walter et al., 1998a). Upon addition of magnesium, or other divalent metal ions, the stacking is preserved but the axes rotate to form an antiparallel structure, allowing the intimate association between the loops. The folding of the complete ribozyme is induced by the cooperative binding of magnesium ions, with a transition midpoint of approximately 30 μM under our working conditions.

The folding of the hairpin ribozyme clearly involves both the four-way junction and the loops. The isolated junction undergoes an ion-induced transition to an antiparallel structure, but this requires a 100-fold higher magnesium ion concentration compared to the complete ribozyme, and the folding lacks the cooperativity observed in the ribozyme (Walter et al., 1998a). Most mutations in the loops that significantly impair cleavage, such as G + 1A (Chowrira et al., 1991) and A10U (Wilson et al., 2001), exhibit folding properties that are much closer to those of the isolated junction (Zhao et al., 2000; Wilson et al., 2001). The folding of the ribozyme in the absence of the four-way junction is even more impaired (Walter et al., 1999; Zhao et al., 2000); the “hinged” form of the ribozyme that lacks the C and D arms of the junction requires a 1,000-fold higher magnesium ion concentration to promote loop-loop contact (Zhao et al., 2000). Thus, both the loops and the junction evidently contribute to the ion-induced folding of the complete ribozyme.

Although both the loop interaction and the folding of the helical junction are likely to involve metal ion binding, a number of questions remain. It is not clear whether this requires site-specific ion binding, or whether general charge screening by a diffuse ion atmosphere is sufficient. The latter case is supported by the observation that the ribozyme is active in molar concentrations of monovalent cations (Murray et al., 1998). However, under physiologically relevant conditions, specific divalent ion binding may be required. If this is so, then a better definition of the location and numbers of bound metal ions is required. To this end, we have carried out detailed studies of folding as a function of monovalent and divalent metal ions, using FRET. We conclude that folding is a complex process that involves stabilization of the active form by a combination of diffuse and site-specific ionic interactions.

RESULTS

Analysis of folding using fluorescence resonance energy transfer

Ion-induced conformational transitions of the hairpin ribozyme have been mainly studied using FRET (Murchie et al., 1998; Walter et al., 1998a, 1998b, 1998c, 1999; Zhao et al., 2000; Wilson et al., 2001). In this approach, donor and acceptor fluorophores are attached to the 5’ termini of selected arms, and energy transfer between them is measured as a function of added metal ion concentration. The efficiency of energy transfer ($E_{FRET}$) depends inversely on the separation between the fluorophores ($R$) according to Förster (1948),

$$E_{FRET} = 1/(1 + (R/R_0)^6)$$

where $R_0$ is the characteristic Förster distance at which $E_{FRET} = 0.5$. For a given vector, a junction will comprise one strand labeled with fluorescein (donor), one with Cy3 (acceptor) and two unlabeled strands (Fig. 1). In the junction form of the hairpin ribozyme, there are six distinct end-to-end distances that may be analyzed. Each of these can be labeled in two directions, giving 12 possible vectors for study.

In this work, we have utilized those vectors that exhibit a pronounced shortening on ion-induced folding, corresponding to the distances between the A and B arms, and between the C and D arms. For the majority of the work, we have concentrated on the shortening of the BA vector, that is, the species in which helices B and A carry fluorescein and Cy3, respectively. For all experiments, a 2'-deoxyribose substitution at A-1 was present to prevent ribozyme cleavage.

FIGURE 1. The hairpin ribozyme. A: The base sequence of tobacco ringspot virus satellite (–) RNA in the region containing the hairpin ribozyme (Hampel & Trizcz., 1989), with the site of cleavage indicated by an arrow. The four helical arms are labeled A and B (containing the A and B loops, respectively), continuing C and D in a circular manner. Some specific nucleotides are numbered (Chowrira et al., 1991). B: Schematic example of a fluorophore-labeled vector used in the FRET experiments. The strands are named with lower case letters, placed as the FRET experiments.
Magnesium-ion-induced folding of the isolated four-way junction of the hairpin ribozyme

To begin with, we studied the isolated four-way junction of the hairpin ribozyme, where the A- and B-loops have been converted to regular duplex by complementation. This is a perfect 4H (Lilley et al., 1995) junction. In this form, there is no possibility for loop–loop interaction, and any folding is solely due to the four-way helical junction. The isolated junction adopts a square configuration of coaxially stacked arms in the absence of added metal ions (Murchie et al., 1998; Walter et al., 1998a). Addition of magnesium ions results in rotation of the helical axes into an antiparallel conformation, thereby shortening the length of the BA vector. We have observed the increase in FRET efficiency on titration of the simple junction in TB buffer with magnesium ions over an extended concentration range (Fig. 2A). The data have been fitted with a phenomenological two-state model

$$E_{\text{FRET}} = e_i + \Delta e \cdot \frac{K_1[Mg^{2+}]^n}{1 + K_1[Mg^{2+}]^n}$$  (2)

where $e_i$ is the FRET efficiency in the absence of added metal ions, $\Delta e$ is the change in FRET on addition of magnesium ions, $K_1$ is an apparent association constant for magnesium ions and $n$ is a Hill coefficient. In applying this model, it is assumed that an equilibrium exists between the structure with approximately perpendicular axes and the folded antiparallel structure (Walter et al., 1998a), and that there is a rapid exchange between these two states, with an insignificant contribution to the measured FRET from transient intermediate structures.

The most simple case of binding by a single magnesium ion ($n = 1$) clearly does not fit the data. Instead, the magnesium-ion-induced folding exhibits anticooperativity ($n = 0.5$). The binding of two or more magnesium ions specifically at the four-way junction could explain the observed anticooperativity. However, at least three binding sites are required to fit adequately a model incorporating specific binding sites (Equation 4, see below), which seems unlikely. An alternative explanation was suggested by the observation of anticooperativity in the diffuse binding of divalent cations by duplex nucleic acids (Krakauer, 1971; Sander & Ts'o, 1971). It seemed possible that diffuse magnesium ion binding could be contributing to the folding in the relatively low ionic strength TB buffer as the antiparallel conformation of the folded junction brings the two stacked arms into close proximity, a conformation that may only be stable with substantial screening of the negative charge of the phosphate groups. In a low-ionic-strength buffer, this screening would be effected by the diffuse binding of magnesium ions. To investigate this possibility, we performed titrations in solutions containing increasing concentrations of sodium chloride, conditions which reduce the energetic contribution of diffuse magnesium ion binding (Record et al., 1976; Bukhman & Draper, 1997).

As expected, the $[\text{Mg}^{2+}]_{1/2}$ (the magnesium ion concentration at which the transition is half complete, given by $K_1^{-1/n}$) increases with increasing ionic strength (Fig. 2B, Table 1). Importantly, the value of $n$ also increases to unity. No cooperativity is observed in the

\[ \text{FIGURE 2. Ion-induced folding of the simple four-way junction derived from the hairpin ribozyme. Folding has been followed by the increase in FRET efficiencies for the BA vector, measured in TB buffer (with or without added sodium chloride) at 4°C. FRET efficiencies plotted as a function of magnesium ion concentration (on a logarithmic scale). A: Folding in the absence of added monovalent metal ions. The data (filled squares) have been fitted using a simple binding model (Equation 2). The Hill coefficient was fixed at } n = 1 \text{ (broken line), or allowed to vary (solid line) whereupon a value of } n = 0.53 \text{ was obtained. B: Folding in the presence of various concentrations of sodium ions. The lines show the best fits of Equation 2 to the individual data sets, and the estimated parameters are collated in Table 1. Data were collected in the presence of the following sodium ion concentrations: 0 mM (filled squares), 20 mM (open squares), 50 mM (filled circles), 100 mM (open circles), 200 mM (filled triangles), and 500 mM (open triangles).} \]
magnesium ion-binding isotherm in solutions containing 100 mM NaCl or higher, conditions where the energetic contribution of nonspecific magnesium ion binding is less significant (Record et al., 1976; Buhkman & Draper, 1997). The simplest interpretation of these data is that there is a single critical magnesium ion bound at the four-way junction, but that effective folding also requires a significant ionic strength to provide general charge neutralization.

**Magnesium-ion-induced folding of the complete hairpin ribozyme**

We have shown previously that the folding of the complete hairpin ribozyme in its natural junction form requires the cooperative binding of divalent metal ions (Murchie et al., 1998; Walter et al., 1998b). In this work, we have reexamined the folding over an extended range of magnesium ion concentrations. As with the simple junction, we have analyzed the FRET efficiency of the BA vector as a function of ionic concentration (Fig. 3A). Equation 2 does not adequately describe the data, as shown by the systematic distribution of residuals. Presenting the data in the form of a Hill plot (Hill, 1910; Fig. 3B) reveals two distinct phases. Highly cooperative magnesium ion binding occurs at low ion concentrations, reaching a Hill coefficient of approximately 3, whereas we observe anticooperative binding ($n < 1$) at higher concentrations. Accordingly, we have added another term to Equation 2:

$$E_{\text{FRET}} = e_i + \Delta e_1 \cdot \frac{K_1 \left[ \text{Mg}^{2+} \right]^n}{1 + K_1 \left[ \text{Mg}^{2+} \right]^n} + \Delta e_2 \cdot \frac{K_2 \left[ \text{Mg}^{2+} \right]}{1 + K_2 \left[ \text{Mg}^{2+} \right]}$$  \hspace{1cm} (3)

where $e_i$ is the FRET efficiency in the absence of added metal ions, $\Delta e_1$ and $\Delta e_2$ are changes in FRET efficiency on addition of magnesium ions, $K_1$ and $K_2$ are apparent association constants for magnesium ions, and $n$ is the Hill coefficient for the transition corresponding to $K_1$. In using this model, we make the same assumptions that applied to the hairpin junction. It should be noted that Equation 3 could also apply to a three-state system, where $e_i + \Delta e_1$ is the FRET efficiency of an intermediate state and $e_i + \Delta e_1 + \Delta e_2$ is the FRET efficiency of the folded state. As can be seen in Figure 3A, this leads to a substantially better agreement with the experimental data. The midpoint for the cooperative phase of magnesium ion binding of 38 $\mu$M is in good agreement with that of 30 $\mu$M determined previously for titrations over a narrower range of magnesium ion concentrations (Walter et al., 1998a). Introduction of cooperativity in the term containing $K_2$ led to no significant improvement in the fit for these data or those presented below (not shown), and we have therefore left this part noncooperative.

Although the above model adequately fits the data, it is overparameterized, showing high covariation between all parameters, and especially between $K_1$ and $n$. Analysis of the uncertainty in the estimation of parameters by the support plane method (Johnson & Frasier, 1985) demonstrates that these two parameters can take a large range of compensating values that combine to produce a well-defined [Mg$^{2+}$]$^{1/2}$ (Table 2). For this reason, we considered an explicit ligand binding model applicable to a two-state system, the Adair equation (Weber, 1992):

$$E_{\text{FRET}} = e_i + \Delta e \cdot \frac{\sum_{j=0}^{N} j \cdot N C_j \cdot K_j \cdot [\text{Mg}^{2+}]^j}{\sum_{j=0}^{N} N C_j \cdot K_j \cdot [\text{Mg}^{2+}]^j}$$  \hspace{1cm} (4)

where $N$ is the number of binding sites, $K_j$ is the extrinsic association constant for the binding of the $J$th ion, and $N C_j$ is the number of combinations of $J$ from $N$ (given by $N C_j = N!/(J!(N - J)!)$). However, five binding sites were required to obtain a fit close to that obtained with the preceding model (Table 2), with the Adair model.

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**TABLE 1. Magnesium-ion-induced folding of the isolated four-way junction derived from the hairpin ribozyme.**

<table>
<thead>
<tr>
<th>[NaCl] mM</th>
<th>$e_i$</th>
<th>$\Delta e$</th>
<th>$K_1$</th>
<th>$n$</th>
<th>$K_2$</th>
<th>$\chi^2$</th>
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<td>0</td>
<td>0.076 ± 0.004</td>
<td>0.33 ± 0.01</td>
<td>22 ± 5</td>
<td>0.53 ± 0.03</td>
<td>0.0029</td>
<td>7.3e-6</td>
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<td>20</td>
<td>0.111 ± 0.001</td>
<td>0.273 ± 0.005</td>
<td>50 ± 8</td>
<td>0.77 ± 0.03</td>
<td>0.0062</td>
<td>6.5e-6</td>
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<tr>
<td>50</td>
<td>0.115 ± 0.002</td>
<td>0.262 ± 0.007</td>
<td>68 ± 16</td>
<td>0.89 ± 0.04</td>
<td>0.0087</td>
<td>1.3e-5</td>
</tr>
<tr>
<td>100</td>
<td>0.124 ± 0.001</td>
<td>0.246 ± 0.008</td>
<td>89 ± 26</td>
<td>1.05 ± 0.05</td>
<td>0.014</td>
<td>1.4e-5</td>
</tr>
<tr>
<td>200</td>
<td>0.134 ± 0.001</td>
<td>0.221 ± 0.008</td>
<td>66 ± 19</td>
<td>1.06 ± 0.05</td>
<td>0.020</td>
<td>8.2e-6</td>
</tr>
<tr>
<td>500</td>
<td>0.148 ± 0.001</td>
<td>0.22 ± 0.02</td>
<td>44 ± 20</td>
<td>1.08 ± 0.09</td>
<td>0.030</td>
<td>1.5e-5</td>
</tr>
</tbody>
</table>

\(^a\)Estimated parameters and asymptotic standard deviations obtained by fitting Equation 2 to the data presented in Figure 2. Folding was followed by the increase in FRET efficiencies for the BA vector, measured in TB buffer (with or without added sodium chloride) at 4 °C.

\(^b\)The units for the apparent association constant are M$^{-1/2}$, where $n$ is the value of the parameter listed for a given data set.
failing to fit the rapid change in FRET at low magnesium concentrations. When applied to an indirect measure of binding, such as folding of the hairpin ribozyme, this model assumes that the binding of each of N ligands (i.e., metal ions) has an effect on the measurement equivalent to 1/N of the total, an unlikely assumption.

A better fit requiring fewer parameters was obtained by adding another term to Equation 4:

\[
E_{\text{FRET}} = e_i + \frac{\Delta e_i}{N_1} \left[ \sum_{j=0}^{N_1} N_i C_{i,j} K_{1,j} [\text{Mg}^{2+}]^j \right] + \frac{\Delta e_2}{N_2} \left[ \sum_{j=0}^{N_2} N_2 C_{2,j} K_{2,j} [\text{Mg}^{2+}]^j \right]
\]

with the parameters defined as for the simple Adair equation. As for Equation 3, Equation 5 could apply to either a two- or three-state system. The simplest such model that gives a good fit to the data has \( N_1 = 3 \) and \( N_2 = 1 \). The fit using this model is almost as good as that using Equation 3, and does not suffer from the high covariation of parameters. Furthermore, \( K_{2,1} \) and \( K_{2,2} \) converge to zero, eliminating their terms from the equation. Consequently, this model is equivalent to Equation 3 with \( n = 3 \), and the estimates of parameter values obtained using the two equations are very similar.

The effect of sodium ions on the magnesium-ion-induced folding of the hairpin ribozyme

We have sought to disentangle the relatively complex nature of the magnesium-ion-induced folding of the complete ribozyme by studying the process in solutions containing constant sodium ion concentrations, observing the FRET efficiency of the BA vector as before. At low sodium ion concentrations, up to 100 mM, there is little change in the shape of the magnesium binding isotherm, although there is an increase in the magnesium ion concentration required to achieve folding (Fig. 4A). In the presence of higher concentrations of sodium ions, we observe a markedly different pattern (Fig. 4B). Even in the absence of magnesium ions, the ribozyme is significantly folded, with an \( E_{\text{FRET}} \sim 0.3 \) at...
TABLE 2. Magnesium-ion-induced folding of the hairpin ribozyme, comparison of models.\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Number of parameters(^b)</th>
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<th>Second K apparent</th>
<th>(\chi^2)</th>
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<td>(K^c)</td>
<td>(n)</td>
<td>([\text{Mg}]_{1/2})</td>
<td>(K^d)</td>
</tr>
<tr>
<td></td>
<td>(M^{-1/n})</td>
<td>(M)</td>
<td>(M)</td>
<td>(M)</td>
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<td>3.8e15</td>
<td>3.5 ± 0.3</td>
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<tr>
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\(\chi^2\) for Equation 5

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\(\chi^2\) for Equation 5

\(^a\) Estimated parameters and asymptotic standard deviations obtained by fitting various models to the data presented in Figure 3A. Folding was followed by the increase in FRET efficiencies for the BA vector, measured in TB buffer at 4°C.

\(^b\) The number of parameters that were varied in the fitting procedure.

\(^c\) The units for the apparent association constant are \(M^{-1/n}\), where \(n\) is the value of the parameter listed for a given data set.

\(^d\) The confidence intervals given for each parameter were determined by a support plane analysis.

\(^e\) Parameters with a value of zero were held constant during the fitting procedure. If permitted to vary, these parameters converged to zero.

\(^f\) For Equation 5, data under \(K_3\) and \(K_4\) refer to \(K_{1,3}\) and \(K_{2,1}\) respectively. The units of \(K_{2,1}\) are \(M^{-1}\).
500 mM sodium ions. A further shortening of the BA vector is induced by the addition of magnesium ions in the 10 mM range of concentration. Table 3 lists the parameters determined by fitting various models to the data measured in different background monovalent ion concentrations. It is apparent that at high concentrations of sodium ions, the cooperative binding phase has disappeared, and that more simple models adequately describe the data.

**Folding of the hairpin ribozyme induced by sodium ions**

The relatively high FRET efficiency observed in 500 mM sodium ions in the absence of magnesium ions demonstrates that sodium ions can induce at least a partial folding of the hairpin ribozyme. We have therefore examined the folding of the ribozyme induced by sodium ions alone, using the BA vector as before. Sodium ions clearly induce cooperative folding of the hairpin ribozyme, with the FRET efficiency reaching a plateau of 0.3 by a concentration of 1 M, significantly lower than the FRET efficiency observed at high magnesium ion concentrations (Fig. 5). Equation 2 provides an adequate fit to the data, giving values of $[\text{Na}^+]_{1/2} = 240$ mM and $n = 2.8$.

**Analysis of ion-induced folding by means of other fluorescent vectors**

The ion-induced folding of the complete ribozyme exhibits two distinct phases, in contrast to that of the simple four-way junction. Because both the junction and the loops clearly contribute to the folding process in the ribozyme (Murchie et al., 1998; Walter et al., 1999; Zhao et al., 2000), the transition could be expected to be more complex. We wondered to what extent the changes in FRET efficiency might reflect local versus global folding of the ribozyme.

We therefore constructed two new vectors. The first was the vector AB, in which the fluorescein donor and Cy3 acceptor were simply exchanged in position. Because the environment of the two fluorophores is very different (Norman et al., 2000), the efficiency of energy transfer could respond differently if one or the other change was essentially local. The second vector DC places the fluorophores on the two arms lacking the loops, and therefore reports on the structure of a distinct part of the molecule. The two vectors were studied as a function of magnesium ion concentration as before (Fig. 6), and analyzed independently using Equation 5 (Table 4). Both vectors give parameters similar to those determined for the BA vector.
TABLE 3. Magnesium-ion-induced folding of the hairpin ribozyme, effect of monovalent cations.

<table>
<thead>
<tr>
<th>[NaCl] mM</th>
<th>N&lt;sup&gt;a&lt;/sup&gt;</th>
<th>e&lt;sub&gt;i&lt;/sub&gt;</th>
<th>Δe&lt;sub&gt;i&lt;/sub&gt;</th>
<th>K&lt;sub&gt;i,N&lt;/sub&gt;</th>
<th>[Mg]&lt;sup&gt;1/2&lt;/sup&gt;</th>
<th>[Mg]&lt;sup&gt;1/2&lt;/sup&gt;</th>
<th>χ&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>First phase</td>
<td>Second phase</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>0.074 ± 0.002</td>
<td>0.158 ± 0.004</td>
<td>1.7 ± 0.1</td>
<td>3.9e-5</td>
<td>0.130 ± 0.004</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>0.104 ± 0.003</td>
<td>0.17 ± 0.01</td>
<td>8.2 ± 0.8</td>
<td>1.1e-4</td>
<td>0.13 ± 0.01</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>0.110 ± 0.002</td>
<td>0.156 ± 0.009</td>
<td>12.9 ± 0.08</td>
<td>2.0e-4</td>
<td>0.11 ± 0.01</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>0.164 ± 0.003</td>
<td>0.140 ± 0.008</td>
<td>9.4 ± 1.0</td>
<td>2.2e-4</td>
<td>0.076 ± 0.009</td>
<td>620 ± 200</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>0.118 ± 0.002</td>
<td>0.105 ± 0.007</td>
<td>3.1 ± 0.3</td>
<td>3.2e-4</td>
<td>0.158 ± 0.008</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>0.194 ± 0.002</td>
<td>0.040 ± 0.018</td>
<td>5.8 ± 1.8</td>
<td>4e-4</td>
<td>0.14 ± 0.02</td>
<td>6.5 ± 1.6</td>
</tr>
<tr>
<td>500</td>
<td>1</td>
<td>0.292 ± 0.001</td>
<td>0.043 ± 0.018</td>
<td>1.0 ± 0.6</td>
<td>1.0e-3</td>
<td>0.047 ± 0.017</td>
<td>91 ± 65</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimated parameters and asymptotic standard deviations obtained by fitting Equation 5 to the data presented in Figure 4. Folding was followed by the increase in FRET efficiencies for the BA vector, measured in TB buffer (with or without added sodium chloride) at 4°C, except where noted.

<sup>b</sup>N is the number of binding sites assumed in the first folding phase, that is, N<sub>i</sub> in Equation 5. N<sub>i</sub> = 1 in each case.

<sup>c</sup>K<sub>i,N</sub> is the relevant association constant of the first phase, having units of M<sup>-N</sup>, where N is as listed for a given concentration of NaCl.

<sup>d</sup>Measured at 25°C.

We have also analyzed a vector that is based on a different pair of fluorophores. We constructed an alternative AB vector, using Cy3 as the donor and Cy5 as the acceptor. Both fluorophores were attached to fully base paired 5’CC termini by three-carbon linkers, and the fluorescence anisotropies of both fluorophores were high (Cy3 = 0.323 ± 0.001 and Cy5 = 0.324 ± 0.001). Thus it is probable that the Cy5 stacks onto the end of the helix in a similar manner to that established for Cy3 (Norman et al., 2000). If both fluorophores are bound to the RNA in a rigid manner, the efficiency of energy transfer will be dependent on the relative orientation of the fluorophores, and not simply on their scalar separation. We have analyzed the FRET efficiency of the AB (Cy3–Cy5) vector as a function of magnesium ion concentration (Fig. 6) using the closely related normalized acceptor ratio ((ratio)<sub>A</sub>; see Materials and Methods). As (ratio)<sub>A</sub> and E<sub>FRET</sub> are linearly dependent, the

![FIGURE 5. Sodium-ion-induced folding of the hairpin ribozyme. Folding has been followed by the increase in FRET efficiency for the BA vector, measured in TB buffer at 4°C. Plot of the change in FRET efficiency (filled circles) as a function of sodium ion concentration (on a logarithmic scale). The solid line shows the best fit to the data of Equation 2. The estimated parameters are e<sub>i</sub> = 0.099 ± 0.004, Δe = 0.208 ± 0.003, K<sub>i</sub> = 56 ± 15, n = 2.8 ± 0.2, and χ<sup>2</sup> = 2.5e-5.](image)

![FIGURE 6. Magnesium-ion-induced folding of the hairpin ribozyme observed by different fluorescent vectors. Folding has been analyzed by the increase in energy transfer for different vectors measured in TB + 100 mM sodium chloride at 4°C. The solid lines show the best fit applying Equation 5 to all four data sets, sharing all association constants, with the estimated parameters collated in Table 4. The following vectors (fluorescein — Cy3 FRET efficiency except where indicated) were used in this analysis: BA (filled circles); AB (filled triangles); DC (filled inverted triangles) and A(Cy3)B(Cy5) as (ratio)<sub>A</sub> value (diamonds).](image)
parameters for ion binding can be directly compared with those for the other vectors. Fitting to Equation 5 gives similar parameters to those above (Table 4).

All four data sets presented in Figure 6 were also analyzed simultaneously in a global fit (Table 4). We found that all sets were satisfied by shared values of $K_{1,3}$ and $K_{2,1}$, which were similar to those obtained independently for each data set. Moreover, the relative change in $E_{\text{FRET}}$ in the two phases was similar, so that $0.37 < \Delta e_1/(\Delta e_1 + \Delta e_2) < 0.50$. Further constraining the global fit such that $\Delta e_1/(\Delta e_1 + \Delta e_2)$ was identical for all data sets produced little change in parameters. The similarity in the titrations obtained for the four different vectors suggest that the change in FRET efficiency measures the global folding of the ribozyme, with little or no contribution from local conformational changes.

**Correlation of magnesium-ion-induced folding with catalytic activity**

We may compare the ion-induced folding of the hairpin ribozyme with its acquisition of catalytic activity. To make a reliable comparison, we have measured folding and cleavage activity under identical conditions, that is, 90 mM Tris borate (pH 8.3) buffer containing 50 mM sodium ions at 25°C (Fig. 7). The profile of FRET efficiency for the BA (fluorescein–Cy3) vector as a function of magnesium ion concentration is closely similar to that observed before (compare with Fig. 4A), and has been fitted using Equation 5 (Table 3).

We have analyzed cleavage activity by the junction form of the hairpin ribozyme using a two-stranded construct (Wilson et al., 2001), comprising a transcribed ribozyme strand together with a chemically synthesized radioactively labeled substrate strand. In this species, the junction-distal helix of arm A can only form 3 bp, and thus the product to the 3′ side of the cleavage should readily diffuse away, reducing the probability of re-ligation. The experiment was carried out under single-turnover conditions, hybridizing substrate (50 nM) with an excess of ribozyme (1 μM), and ini-

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**TABLE 4.** Magnesium-ion-induced folding of the hairpin ribozyme observed by different fluorescent vectors.

<table>
<thead>
<tr>
<th>Vector</th>
<th>$e_1$</th>
<th>$\Delta e_1$</th>
<th>$K_{1,3}$ M$^{-1}$</th>
<th>$[\text{Mg}]_{1/2}$ M</th>
<th>$\Delta e_2$</th>
<th>$K_{2,1}$ M$^{-1}$</th>
<th>$[\text{Mg}]_{1/2}$ M</th>
<th>$\Delta e_1/\Delta e_1 + \Delta e_2$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>0.118 ± 0.002</td>
<td>0.105 ± 0.007</td>
<td>6.1 ± 0.3 e10</td>
<td>3.2 ± 0.3 e10</td>
<td>0.158 ± 0.008</td>
<td>1.2 ± 0.1 e3</td>
<td>8.5 ± 0.4 e3</td>
<td>0.40</td>
<td>7.6e-6</td>
</tr>
<tr>
<td>AB</td>
<td>0.151 ± 0.003</td>
<td>0.115 ± 0.009</td>
<td>2.2 ± 0.3 e10</td>
<td>3.6 ± 0.4 e10</td>
<td>0.15 ± 0.01</td>
<td>1.7 ± 0.2 e3</td>
<td>5.8 ± 0.4 e3</td>
<td>0.44</td>
<td>1.8e-5</td>
</tr>
<tr>
<td>DC</td>
<td>0.118 ± 0.002</td>
<td>0.07 ± 0.01</td>
<td>5.1 ± 0.1 e10</td>
<td>3.0 ± 0.1 e10</td>
<td>0.13 ± 0.01</td>
<td>1.1 ± 0.2 e3</td>
<td>8.8 ± 0.4 e3</td>
<td>0.33</td>
<td>2.4e-5</td>
</tr>
<tr>
<td>AB(Cy3–Cy5)</td>
<td>0.093 ± 0.003</td>
<td>0.13 ± 0.01</td>
<td>4.6 ± 0.6 e10</td>
<td>2.8 ± 0.6 e10</td>
<td>0.13 ± 0.01</td>
<td>6 ± 1 e2</td>
<td>2e-3</td>
<td>0.49</td>
<td>3.4e-5</td>
</tr>
</tbody>
</table>

*Estimated parameters and asymptotic standard deviations obtained by fitting Equation 5 ($N_1 = 3$, $N_2 = 1$) to the data presented in Figure 6. Folding has been analyzed by the increase in energy transfer for different vectors measured in TB + 100 mM NaCl at 4°C.

Global fit of all four data sets, with $K_{1,3}$ and $K_{2,1}$ as global parameters. This fit is plotted in Figure 6.

Range of values. The actual values for each vector are BA: 0.42, AB: 0.50, DC: 0.37, and AB(Cy3–Cy5): 0.39.

Global fit of all four data sets, with $K_{1,3}$, $K_{2,1}$, and $(\Delta e_1/\Delta e_1 + \Delta e_2)$ as global parameters.

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**FIGURE 7.** Comparison of cleavage activity with the extent of ion-induced folding for the hairpin ribozyme. Both properties were measured under the same conditions of TB buffer plus 50 mM sodium chloride at 25°C. Folding has been followed by the increase in FRET efficiency for the BA vector (closed circles). The data have been fitted to Equation 5 (line), with the estimated parameters collated in Table 3. Cleavage rates (open squares) were measured as described in the text, and the values of observed rate constants plotted as a function of magnesium ion concentration. Measurements were made in triplicate and the mean values plotted with standard errors.
tiating the reaction by addition of magnesium ions at a range of concentrations. In common with other investigations of this ribozyme (Esteban et al., 1997), biphasic progress curves were obtained. We have therefore measured the initial rate of reaction, and these are plotted as a function of magnesium ion concentration in Figure 7. It is clear that the observed rate constants closely follow the extent of folding. The midpoint occurs at approximately 400 \( \mu \text{M} \) magnesium ions, and the maximum rate is 0.13 min\(^{-1}\).

DISCUSSION

Like most branched RNA species, the hairpin ribozyme undergoes a conformational transition upon the addition of metal ions; the change in structure is induced by the binding of one or more metal ions. The polyelectrolyte character of RNA leads to a dominance of electrostatic interactions, and thus charge screening by counterions will be very important. Moreover, folding of branched RNA species is likely to generate electronegative clefts with significantly altered binding characteristics, and association with ions can thus change the position of the conformational equilibrium. However, the binding of ions to nucleic acids can, in principle, occur in two different forms (reviewed in Lilley & Clegg, 1993; Draper & Misra, 1998; Misra & Draper, 2001). First, the interaction can be largely electrostatic, leading to charge neutralization by a diffuse cloud of ions (Eisenman, 1962). A given site of high electrostatic potential could lead to a high occupancy by cations, and bind divalent in preference to monovalent cations, but a given ion will be in rapid exchange with those of the bulk. These ions retain their full solvation spheres and their interaction with sites on the nucleic acid is essentially Coulombic. Secondly, a limited set of metal binding events can involve inner-sphere interaction with specific ligands on the RNA, forming a defined local geometry, and is characterized by a much slower exchange rate with ions in the bulk. Although this is well exemplified by sodium ions bound along the axis of a guanine tetrplex structure (Laughlan et al., 1994), it is more commonly observed for divalent metal ions such as magnesium (e.g., Cate et al., 1996). In some structures, examples of both types of binding can be seen within the same molecule (e.g., Ennifar et al., 1999) Our results suggest that both types of binding are responsible for the folding of the hairpin ribozyme.

We have first examined the ion-induced folding of the isolated four-way RNA junction, derived by complementing the loops of the hairpin ribozyme. Even this simple junction exhibits two distinct types of ion binding, as observed indirectly by the analysis of the RNA folding by FRET. Our data on folding in a low ionic strength background of monovalent ions exhibit anti-cooperative binding of magnesium ions with a Hill coefficient \( n < 1 \). This is typical for diffuse binding of magnesium ions by nucleic acids (Krakauer, 1971; Sander & Ts'o, 1971), and suggests that charge screening by an ion atmosphere is required to allow the closer approach of the helical arms as the axes rotate into an antiparallel conformation. At higher concentrations of monovalent ions, noncooperative binding of magnesium ions was observed. In the presence of \( \approx 100 \text{ mM} \) sodium ions, addition of magnesium ions induced rotation into the antiparallel junction conformation with \( n = 1 \). The folding of the junction induced by a relatively low magnesium ion concentration in the presence of 500 mM monovalent ions is strong evidence for site binding of the magnesium ion (Bukhman & Draper, 1997). Under these conditions, the overall charge on the helical arms is significantly reduced by the diffuse binding of sodium ions, and the electrostatic interactions between divalent cations and duplex RNA will be relatively weak (Record et al., 1976; Bukhman & Draper, 1997). Although magnesium ions will tend to displace sodium ions from the diffuse cloud of ions, this diffuse binding of magnesium will not significantly affect the energetics of folding, which suggests that the folding is induced by the site-binding of magnesium ions. The simplest model consistent with the Hill coefficient is the noncooperative binding of a single magnesium ion at or near the four-way junction. No ions were seen coordinated to the junction in the crystal structure of the ribozyme (Rupert & Ferré-D’Amaré, 2001), but this may be due to the very different conditions of crystallization. Furthermore, the local conformation of the junction may well be significantly altered in the context of the complete ribozyme, together with its ion binding properties.

The folding of the complete ribozyme is more complex, and involves a number of binding events. At low concentrations of monovalent cations, our magnesium titrations contain two distinct phases. An initial cooperative binding phase produces a shortening of the end-to-end distance of both the BA and DC vectors. This is followed by noncooperative binding at a 10-fold higher concentration, which gives a further reduction in the end-to-end distance. Neither a phenomenological model (Equation 2) nor an explicit binding model (Equation 4) gives a good fit to data obtained at low monovalent ion concentrations. In both cases, an additional term is needed to account for the additional binding at higher magnesium concentrations. Although Equation 3 is overparameterized, with the values for \( K_1 \) and \( n \) covarying, the model still gives robust estimates of the midpoint magnesium ion concentration of each phase. When fitting Equation 5 to sets of data, it was found that \( K_{1,1} \) and \( K_{1,2} \) usually converged to zero, and no significant improvement in the fit was obtained by fixing \( K_{1,1} = K_{1,2} = 0 \). The elimination of these two association constants implies total cooperativity of binding. That is, no intermediates exist, only the unbound state and the fully bound state. In this case, Equation 5 is equiv-
alent to Equation 3 with \( n = 3 \), and this is the simplest model that gives a satisfactory fit to the data.

The initial cooperative phase of ribozyme folding can be accomplished by relatively high concentrations of monovalent metal ions. It is therefore likely to involve the diffuse binding of ions. Thus the value of \( n = 3 \) obtained at low monovalent ion concentrations suggests that the binding of at least three magnesium ions contributes to this cooperative phase, and is consistent with the good fit obtained using Equation 5. However it is surprising that a similar Hill coefficient is obtained for the folding of the ribozyme by sodium ions. Given the electrostatic nature of diffuse binding, it might be expected that more monovalent than divalent cations would be required to stabilize the folded structure. Therefore, we propose that the order of the Hill coefficient (\( n \)) and the number of sites in the Adair equation (\( N \)) do not measure the number of cations bound, but rather the number of regions of high negative potential created on folding the ribozyme. It should be remembered that we do not measure binding of ions directly in our experiments, but rather the conformational consequences of binding. As the loops of the ribozyme come into contact it is likely that regions at the interface have a greater negative potential than the corresponding regions of the individual loops. Each of these newly created electrosensitive clefts will result in an increase and/or redistribution of diffusely bound ions, stabilizing the overall interaction between the loops and leading to the observed dependence on cooperative binding. In other words, all the sites of high negative potential exist, or none, as was suggested by the fitting of Equation 3 to our data.

The second phase of the ribozyme folding can be well fitted without inclusion of cooperativity. It is not observed with monovalent ions, and occurs in a high background concentration of sodium ions. Thus the data are consistent with the noncooperative, site-specific binding of a magnesium ion. Although site-specific binding was observed in the isolated junction derived from the ribozyme, we believe that this is not the same site that becomes bound in the second folding phase of the complete ribozyme. The interaction constrains the conformations available to the A and B arms of the ribozyme and serves to direct their interaction. Moreover, the interaction between the loops will stabilize the junction in its folded form. As might be expected from such reciprocity, the first phase of binding occurs at magnesium ion concentrations that are orders of magnitude lower than that observed for either the isolated junction ([Mg]_1:2 = 3 mM), or the hinged form of the ribozyme lacking the four-way junction ([Mg^2+]_1:2 \( \approx 20 \) mM; Zhao et al., 2000). Thus, it seems highly likely that the folding of the junction is coupled to the initial, cooperative phase of the folding of the complete ribozyme. However, our data do not permit us to determine whether site-specific magnesium ion binding at the junction contributes to the folding of the ribozyme. If the ribozyme junction has the same structure as the isolated junction, then a magnesium ion should bind at the junction and contribute to the stability of the folded ribozyme. But the folding of the ribozyme by monovalent cations demonstrates that site-specific binding at the junction is not essential.

We note that both phases of the folding process involve the shortening of both the B–A and the C–D distances. The efficiencies of energy transfer observed for the four vectors indicate that they move different distances, as would be expected. Yet the similarity of each magnesium ion titration, as shown by the global fit of Equation 5 to the four data sets, is convincing evidence that the local environment of the fluorophores does not significantly affect our data. Thus both the initial diffuse binding and the subsequent site-specific binding events involve the global structure of the complete ribozyme, apparently in a kind of scissorlike rotation. This implies a coordination between the loop–loop interaction and the four-way junction, consistent with earlier studies showing that both structures are essential for efficient folding of the ribozyme (Murchie et al., 1998; Walter et al., 1999; Zhao et al., 2000).

We have observed a close correlation between the metal ion dependence of folding and cleavage activity, suggesting that the rate of cleavage is determined by the proportion of folded ribozyme present in solution. Thus folding could be the major role for the metal ions, possibly even the only role. This is consistent with the activity of the ribozyme in other cations, including hexammine cobalt (III) (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997), aminoglycoside antibiotics (Earnshaw & Gait, 1998) and even high concentrations of monovalent metal ions and ammonium ions (Murray et al., 1998). If we assume a two-state folding model, we note that the extent of folding in 0.5 mM sodium ions is similar to that at 100 \( \mu \)M magnesium ions. On the other hand, if the folding is more complex, then the structure achieved in sodium ions is nevertheless competent for cleavage activity.

A number of metal ion binding sites have been observed in the hairpin ribozyme and its components by crystallography (Rupert & Ferré-D’Amaré, 2001) and NMR (Butcher et al., 2000). A further site has been observed to bind terbium (III) ions in solution (Walter et al., 2000). Our present data provide no information on the location of any metal ions responsible for the folding of the ribozyme. However, we speculate that the specific magnesium ion binding observed in our folding experiments may occur at the site where calcium ions were observed making inner-sphere interactions with loop B (Rupert & Ferré-D’Amaré, 2001). This site is on the opposite face of loop B to that which interacts with loop A and may, therefore, be independent of the cooperative folding phase.

In summary, full folding of the hairpin ribozyme requires two different metal ion binding events (Fig. 8).
FIGURE 8. Scheme to illustrate the two-step ion-binding model for the folding of the hairpin ribozyme. The first stage of folding is cooperative, and is induced by the diffuse binding of cations in at least three regions. It occurs with a midpoint of 38 μM for magnesium ions in TB buffer, but can also be induced by high concentrations of sodium ions. Site-specific binding of a magnesium ion at the junction may also contribute to this cooperative folding. In contrast, the second stage can only be induced by divalent metal ions; monovalent ions are unable to bring about this transition, which is therefore likely to be due to site-specific binding. This can be modeled adequately without the inclusion of cooperativity in binding. The locations of ions in this schematic figure is not intended to be interpreted in a literal way.

The first, cooperative, phase can be achieved by the diffuse binding of metal ions. The second phase requires the noncooperative site binding of magnesium ions.

MATERIALS AND METHODS

Chemical synthesis of RNA-containing oligonucleotides

Oligonucleotides were synthesized using phosphoramidite chemistry (Beaucage & Caruthers, 1981) implemented on an Applied Biosystems 394 DNA/RNA synthesizer. RNA was synthesized using ribonucleoside phosphoramidites with 2′-O-t-butyldimethylsilyl (t-BDMS) protection (Hakimelahi et al., 1981; Perreault et al., 1990; PrOligo). 2′-deoxyribonucleosides were introduced at positions indicated in the text. Fluorescein (PE-ABI), Cy3, and Cy5 (Glen Research) were coupled to the 5′ termini as phosphoramidites. Oligoribonucleotides were deprotected in 25% ethanol/ammonia solution at room temperature for 24 h (Cy5-labeled), or at 55 °C for 5 h (all others), and evaporated to dryness. Oligoribonucleotides were redissolved in 0.5 mL 1 M tetrabutylammonium fluoride (Aldrich) in tetrahydrofuran to remove t-BDMS 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 oligoribonucleotides were purified by gel electrophoresis in 20% polyacrylamide containing 7 M urea. Bands were excised, and the oligoribonucleotides were electroeluted into 8 M ammonium acetate, and recovered by ethanol precipitation. Fluorescently labeled oligoribonucleotides were further purified by reversed-phase HPLC. Samples were dissolved in 100 mM triethylammonium acetate, pH 7, and applied to a C18 reversed-phase column (μ Bondapak, Waters). RNA was eluted with a linear gradient of acetonitrile, 100 mM triethylammonium acetate (pH 7) with a flow rate of 1 mL/min. The peak fractions were evaporated to dryness, redissolved in water, and ethanol precipitated.

Construction of hairpin ribozyme species for FRET analysis

Fluorescent hairpin and junction species for FRET studies were constructed by incubation of a mixture of stoichiometric quantities of one donor-labeled (normally fluorescein, but Cy3 in combination with Cy5) strand, one acceptor-labeled (Cy3 or Cy5) strand, and two unlabeled strands in 90 mM Tris-borate, pH 8.3, 25 mM NaCl for 2 min at 80 °C, followed by slow cooling. The hybridized species were purified by electrophoresis in a 10% polyacrylamide gel at 4 °C for 20 h at 120 V. The buffer system contained 90 mM Tris-borate, pH 8.3, 25 mM NaCl, and was recirculated at >1 L/h. Fluorescent junctions were visualized by illumination using a Dark Reader transilluminator (Clare Chemical Research), the bands were excised, and the RNA electroeluted into 8 M ammonium acetate and recovered by ethanol precipitation. FRET analysis of the various forms of the hairpin ribozyme employed synthetic RNA species (with 5′ fluorophores where appropriate) of the following sequences (all written 5′ to 3′, with deoxyribonucleotides underscored):

a strand:  
CCGCACAGAGAAGUCAACCAGAGAAACACACCGG
b strand:  
CCGGUGGUAAUUAACCGUAGCUGGUACGCCUUGACGU
GGGG;
c strand:  
CCCCACGUCAAGGCGUGGGUGCGGCAAGGUGC;
d strand:  
CCGACCUUCGCCACCCUGACGUCCUGUGCGG.

For the simple four-way junction a modified a-strand sequence was made, resulting in a complementation of the A and B loops:

a′ strand:  
CCGCACAGAGAAGUCAACCAGAGAAACACACCGG.

Fluorescence spectroscopy

Fluorescence spectra were recorded at 4 °C using an SLM-Aminco 8100 fluorimeter, in TB buffer (90 mM Tris.borate, pH 8.3) except where otherwise specified. Spectra were corrected for lamp fluctuations and instrumental variations, as described in Bassi et al. (1997). Polarization artifacts were avoided by setting excitation and emission polarizers crossed at 54.7°. Values of $E_{\text{FRET}}$ were measured using the acceptor normalization method (Murchie et al., 1989; Clegg, 1992) in
Folding the hairpin ribozyme by ions

which an extracted acceptor spectrum \( F_A(v_1,v') \) (excitation at \( v' = 490 \text{ nm} \) for fluorescein or 547 nm for Cy3, emission at \( v_1 \)) is normalized to a second spectrum \( (F(v_2,v')) \) from the same sample excited at a wavelength \( (v'' = 547 \text{ nm} \) for Cy3 or 610 nm for Cy5) at which only the acceptor is excited, with emission at \( v_2 \). This gives the acceptor ratio

\[
\text{(ratio)}_A = \frac{F_A(v_1,v')/F(v_2,v'')}{(\epsilon_D(v')/\epsilon_A(v'')) + (\epsilon_A(v')/\epsilon_A(v''))} \\
\times (\Phi_A(v_1)/\Phi_A(v_2)).
\]  

Subscripts \( D \) and \( A \) refer to donor and acceptor respectively. \( \epsilon_D \) and \( \epsilon_A \) are the molar absorption coefficients at the indicated frequency of donor and acceptor respectively and \( \Phi_A \) is the fluorescent quantum yield of the acceptor. \( d^+ \) is the molar fraction of species labeled with donor fluorophore. \( E_{\text{FRET}} \) may be calculated from \( \text{(ratio)}_A \) because \( \epsilon_D(v')/\epsilon_A(v'') \) and \( \epsilon_A(v')/\epsilon_A(v'') \) are measured from absorption spectra, and \( \Phi_A(v_1)/\Phi_A(v_2) \) is unity when \( v_1 = v_2 \). \( E_{\text{FRET}} \) was not calculated for Cy3–Cy5 energy transfer because \( \epsilon_D(v')/\epsilon_A(v'') \) and \( \epsilon_A(v')/\epsilon_A(v''') \) cannot be accurately evaluated due to impurities in the Cy5 preparation.

Fluorescence anisotropy \( r \) was measured by taking the ratio:

\[
r = \frac{F_{VV} - GF_{VH}}{F_{VV} + 2GF_{VH}}
\]

where \( F \) denotes fluorescence intensity and the subscripts \( V \) and \( H \) refer to fluorescence with vertical and horizontal polarizers respectively, in the order excitation, emission, and \( G \) is the ratio \( F_{HV}/F_{HH} \).

Data analysis

All data analysis was performed using the nonlinear curve fitting routine of ORIGIN 5.0 (Microcal). The quoted errors are the asymptotic standard deviations generated by the fitting routine, which typically underestimate the true uncertainty. Where a better estimate of the uncertainty was required, the range of parameter values giving a satisfactory fit was determined by support plane analysis (Johnson & Frasier, 1985), using a cut-off probability of 0.05 for a significant difference in variance using an \( F \)-test.

Analysis of ribozyme cleavage kinetics

Ribozyme (1 \( \mu \text{M} \)) and [5'-\( ^{32}\text{P} \)]-labeled substrate (50 nM) were incubated together in 50 mM Tris.borate, pH 8.3, containing 50 mM NaCl at 90 °C for 2 min, 37 °C for 60 min, and then stored at 0 °C. Prior to the assay, the complex was incubated at 25 °C for at least 10 min. The cleavage reaction was initiated by addition of MgCl\(_2\) to the required final concentration, and aliquots removed at intervals and the reaction terminated by addition to an equal volume of 20 mM EDTA, 95% formamide, except for higher magnesium ion concentrations where a larger volume was used to ensure an excess of EDTA. Substrate and product were separated by electrophoresis in sequencing gels containing 7 M urea. Product formation was quantified by exposure to storage phosphor screens and phosphorimaging (BAS-1500, Fuji). Initial rates (observed rate constants \( k_{\text{obs}} \)) were determined by taking the gradient of semilogarithmic plots of fraction of product as a function of time.

The substrate strand was chemically synthesized, with the sequence UUCGCGCCACGUACAGCCUGU. The ribozyme strand was prepared by transcription, using the natural sequence GGCCACAGAGAGUCUACCCAGAAACACACGCGUUGUGGUAAUUACCCGUAGCGCAAAAGCGCGUGGCGCGAA.

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