The structure and active site of the Varkud satellite ribozyme

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Abstract

The Varkud satellite ribozyme is the largest of the small nucleolytic ribozymes, and the only one for which there is no crystal structure. It can be divided into a trans-acting ribozyme, consisting of five helices organized by two three-way helical junctions, and a stem-loop substrate with which it interacts, primarily by tertiary interactions. We have determined the global fold of the ribozyme, and the manner by which it interacts with the substrate. The substrate interacts with a cleft formed between helices I1 and VI (organized by the lower helical junction), where it contacts the A730 loop, the probable active site of the ribozyme. Within this loop, there is a critical adenine base (A756) that is a candidate for direct nucleobase participation in the cleavage reaction.

Varkud satellite (VS) ribozyme

The VS ribozyme is one of the small nucleolytic ribozymes that undergoes a site-specific self-cleavage reaction by means of a transesterification reaction arising from the attack of a 2′-hydroxy group to generate 2′,3′-cyclic phosphate and 5′-hydroxy termini [1]. It can also catalyse the reverse ligation reaction [2,3]. The ribozyme is found in the VS RNA that occurs in the mitochondria of Neurospora, which is transcribed from the VS DNA [4].

The functional ribozyme can be readily reduced to a single piece of RNA of 154 nt [5]. The sequence and secondary structure is shown in Figure 1. Stem-loop I contains the cleavage site, and is the substrate of the reaction, while the part comprising helices II to VI is the ribozyme.

Cleavage reaction

In the natural context, the VS ribozyme operates as a single continuous piece of RNA, catalysing a cleavage reaction in cis. However, it can be divided into a trans-acting ribozyme plus substrate system [6]. The trans-acting VS ribozyme is different from analogous forms of the other nucleolytic ribozymes, in that its interaction with the substrate is largely through tertiary contacts. This ribozyme is kinetically very well behaved; the cleavage reaction goes virtually to completion in a single phase (Figure 2) [7]. The reaction can be analysed in terms of the formation of a non-covalent complex between the ribozyme (Rz) and substrate (S), i.e.

\[
Rz + S \xrightleftharpoons{kt}{k_1} Rz \cdot S \xrightleftharpoons{kt}{k_2} Rz \cdot P1 \cdot P2
\]

where P1 and P2 are the products, and \( k_2 \) is the rate constant for the central conversion of the bound substrate into product. It may include conformational changes, such as a rearrangement of secondary structure of the substrate [8], in addition to the chemical step. Usually, the 5′ reaction product should rapidly diffuse away and hence \( k_2 \) can be ignored. The rate of the cleavage reaction may be measured as a function of ribozyme concentration (Figure 2), and the data fitted to the equation:

\[
k_{on} = \frac{(k_2 \cdot [Rz])}{([Rz] + K_{on(app)})}
\]

We have determined that \( K_{on(app)} = 1.0\mu M \) and \( k_2 = 2.0\text{ min}^{-1} \) for the natural VS ribozyme sequence [9].

The global structure of the ribozyme is organized by two three-way helical junctions

Examination of the sequence and secondary structure of the VS RNA (Figure 1) reveals two prominent three-way helical junctions, and experience tells us that these are likely to play a key role in the folding of the ribozyme. The hammerhead and hairpin ribozymes are organized around three- and four-way helical junctions, respectively, and such junctions are clearly important architectural features in nucleic acids [10] that can organize the structure of significant regions of RNA. These junctions were therefore the starting...
point for analysing the structure of the five-helix ribozyme in our studies.

The lower junction (the 2–3–6 junction) connects helices II, III and VI, while the upper junction (the 3–4–5 junction) connects helices III, IV and V. They are connected through their common helix III. We reasoned that if we could determine the global structure of each junction,

**Figure 1**
The sequence of the VS ribozyme

The sequence and deduced secondary structure [29] are shown for the natural cis-acting form. The position of cleavage is indicated by the arrow. The dashed line indicates a proposed tertiary interaction between the loops of helices I and V [13]; this may promote a change in the base pairing of the upper stem of the substrate [8]. The nucleotides comprising the A730 loop are shown in bold type.

**Figure 2**
The VS ribozyme cleavage reaction

(A) Time course of ribozyme cleavage. The schematic shows the division of the natural sequence into a ribozyme plus substrate. Radioactive substrate was incubated with the five-helix ribozyme under single-turnover conditions at 37 °C. The substrate and 5 nt product were separated by gel electrophoresis, and an autoradiograph of the gel is shown. The reaction time points are indicated above the gel. (B) Reaction progress plot for the cleavage of substrate. The line shows a single-exponential function fitted to the experimental data.
and the dihedral angle subtended between them, we would have determined the structure of the ribozyme in overall terms.

The 2–3–6 junction is a 2HS,HS, [11] junct-ion, with single-stranded stretches of five and two nucleotides. We determined the global structure using comparative gel electrophoresis and fluorescence resonance energy transfer [7]. The structure is dependent on the presence of bivalent metal ions. Folding is induced by the non-

Figure 3

The global structure of the VS ribozyme, and its interaction with the substrate

(A) Schematic of the secondary structure of the ribozyme, redrawn to correspond to the global structure deduced in these studies. (B) The structure of the five-helix ribozyme. (C) Parallel-eye stereoscopic view of the substrate stem-loop docked into the model for the complete five-helix ribozyme. The scissile phosphate of the substrate is shown as space-filled.
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co-operative binding of magnesium ions with a $[\text{Mg}^{2+}]_{i}$ of approx. 100 μM, into a conformation in which helices III and VI are co-axially stacked, with an acute angle between helices II and VI. The folding properties of the junction are virtually identical in the context of the complete ribozyme [9]. The local structure of this junction was modelled by homology with one occurring in the 23 S RNA.

The 3–4–5 junction is a HS,HS,HS, junction, with three unpaired sections. Its global structure was determined by the same approaches as for the 2–3–6 junction [12]. In common with the 2–3–6 junction, folding is induced by the non-co-operative binding of magnesium ions, with a $[\text{Mg}^{2+}]_{i}$ of approx. 250 μM. In the folded conformation there is a large angle (most probably due to coaxial stacking) between helices III and IV, whereas helices III and V include an acute angle.

We have analysed the effects of many sequence changes in these two junctions on the cleavage activity of the ribozyme. While some are tolerated, others lead to major impairment of catalytic activity. For example, changes in the bases immediately adjacent to helix I (G768 and A656) resulted in large reduction in cleavage activity [7]. These effects could be correlated with perturbation of the global folding of the ribozyme, and explained in terms of the structure of the junction deduced by homology modelling.

Structure of the complete ribozyme
Using the global structures of the two junctions we can assemble the structure of the complete five-helix ribozyme through the common helix III. The only additional information required is the dihedral angle relating helices II and V, and this was determined to be 75° using a gel electrophoretic technique [12]. On this basis, we have constructed a model for the complete ribozyme folded in the presence of magnesium ions (Figure 3B).

Interaction between substrate and ribozyme
The major binding site for the substrate stem-loop is likely to be the cleft between helices II and VI. This is consistent with two observations. The 3’-end of the substrate stem is linked via three nucleotides to the 5’-end of stem II in the cis-acting form of the ribozyme, and thus these regions of ribozyme and substrate must be relatively close together in the complex. In addition, Collins and co-workers [13] have provided evidence for a loop-loop interaction between the substrate loop and that of stem V. We have manually modelled this structure by docking stem-loop I (taken from a recent NMR structure [14]) into our global model of the five-helix ribozyme (Figure 3C).

Identifying important sequences in the ribozyme
Very extensive nucleotide substitutions have been made over much of the VS ribozyme. The effects of these substitutions on cleavage activity can be divided into a number of groups, as follows.

Base pairs
The secondary structure of the ribozyme is important, but the nature of most individual base pairs is not. Many can be reversed or replaced by a different pair without major loss of activity, so long as a base pair is retained at a given position. Helix IV is virtually completely dispensable, so long as junction 3–4–5 can form stably, and the outer end of helix VI can also be deleted with little reduction in activity.

Bulged bases
Adenine bulges occur in helices II, VI and III. In general, deletion of a given bulge lowers activity significantly, and transfer to the opposite strand is even more deleterious. But, in most cases, substitution of the bulged base by a different nucleotide leads to only a small reduction in activity. These bulged bases are therefore unlikely to play any direct role in cleavage; their role is more probably structural, since such bulges kink the axis of the helix [15–17].

Helical junctions
Important nucleotides exist in both junctions, but, once again, these are likely to have a purely structural role. As discussed above, we have found a good correlation between the effects of changes at these positions on cleavage activity of the ribozyme, and on the folding of the individual junctions.

A730 loop
The internal loop in helix VI that contains A730 exhibited the greatest sensitivity to nucleotide
substitutions; virtually every change in sequence led to a large reduction in cleavage rate.

**Active site of the VS ribozyme**

The A730 loop is very probably the active site of the VS ribozyme. Activity is severely reduced by most sequence changes to any of the four nucleotides comprising the loop, without affecting the global folding of the ribozyme [9]. The cleavage site and the A730 loop are naturally juxtaposed in our model of the ribozyme with the substrate docked into the cleft between helices II and VI (Figure 3). Moreover, the ribozyme retained activity when stem-loop I was reconnected via its 5’ end to the 3’ end of stem I [8], and we have observed that it can also be attached to the end of helix VI (D. A. Lafontaine, unpublished work). It is probable that intimate association between the A730 loop and the substrate creates the local environment in which catalysis of the cleavage reaction occurs. This is strongly reminiscent of the hairpin ribozyme, where interaction between two internal loops of RNA generates the active form of the ribozyme [18-20].

**A special nucleotide**

One nucleotide within the A730 loop appears to be especially important in the cleavage reaction. Any substitution of A756 leads to a reduction in cleavage activity by three orders of magnitude, and this is largely because of a lowered rate of cleavage \(k_c\) rather than impaired substrate binding [9]. Indeed, A756 variants act as inhibitors of the cleavage reaction, with \(K_i\) values in the 3.5 to 4.5 \(\mu\)M range.

Closer examination reveals that the most significant aspect is the nucleobase, and in particular its Watson-Crick face [21]. Thus, although a number of changes to the nucleobase reduce cleavage activity by three orders of magnitude, and this is largely because of a lowered rate of cleavage \(k_c\) rather than impaired substrate binding [9]. Indeed, A756 variants act as inhibitors of the cleavage reaction, with \(K_i\) values in the 3.5 to 4.5 \(\mu\)M range.

We do not know the mechanism by which the ribozyme accelerates the cleavage reaction. However, it is likely that catalysis is multi-factorial, and that the rate enhancement contains components from several of the above processes.

A756 is a candidate for direct nucleobase participation in the cleavage reaction of the VS ribozyme, acting, for example, in general acid-base catalysis. At present, however, there is no unequivocal proof of this, and we have not been able to demonstrate a restoration of activity in an A756 abasic variant by addition of exogenous bases [21]. However, this may be a result of an inaccessibility of the resulting pocket. If the adenine base is to act as a general acid or base at near-neutral pH, its \(pK_a\) will need to be raised since the normal \(pK_a\) of adenine N1 is approx. 3.5. This may well be possible in the highly charged environment of a nucleic acid, and altered \(pK_a\) values of adenine have been measured in the leadzyme by NMR [28].

**Conclusion**

In our current view, the global structure of the VS ribozyme is organized by the two three-way helical
junctions, whose folding is induced by the non-cooperative binding of magnesium ions. The lower junction plays a particularly important role, since this creates the substrate-binding cleft between helices II and VI. The folding of the upper junction places the loop of helix V where it can make a loop–loop interaction with the loop of the substrate, further helping to bind the substrate stem-loop and possibly alter its conformation. The scissile phosphate is brought into juxtaposition with the A730 loop within helix VI, creating the environment in which the cleavage reaction is accelerated. The nucleobase of A756 is made a loop-loop interaction with the loop of the stem-loop and possibly alter its conformation.

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References

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Folding of the td pre-RNA with the help of the RNA chaperone StpA
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Abstract
The td group I intron is inserted in the reading frame of the thymidylate synthase gene, which is mainly devoid of structural elements. In vivo, translation of the pre-mRNA is required for efficient folding of the intron into its splicing-competent structure. The ribosome probably resolves exon–intron interactions that interfere with splicing. Uncoupling splicing from translation, by introducing a non-sense codon into the upstream exon, reduces the splicing efficiency of the mutant pre-mRNA. Alternatively to the ribosome, co-expression of genes that encode proteins

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