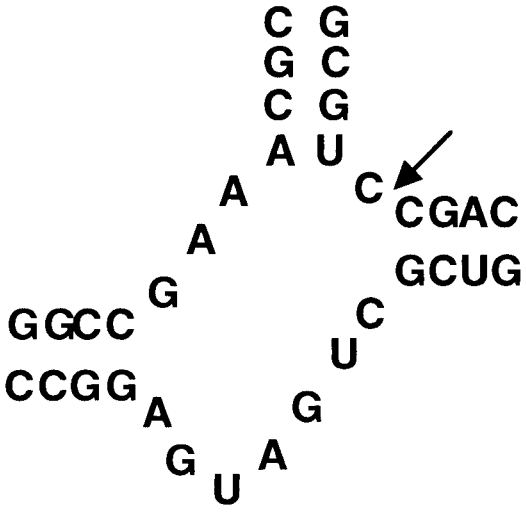
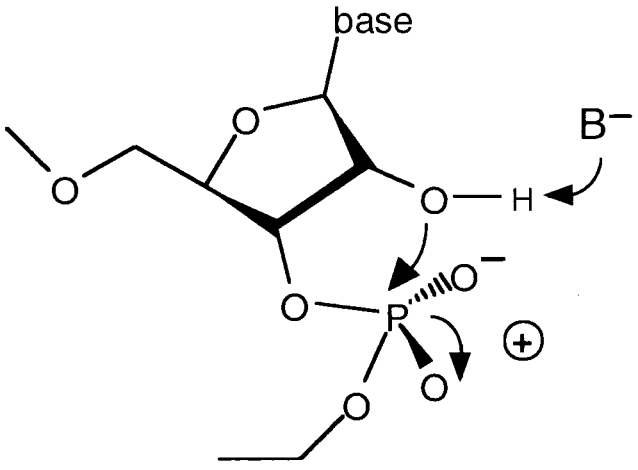
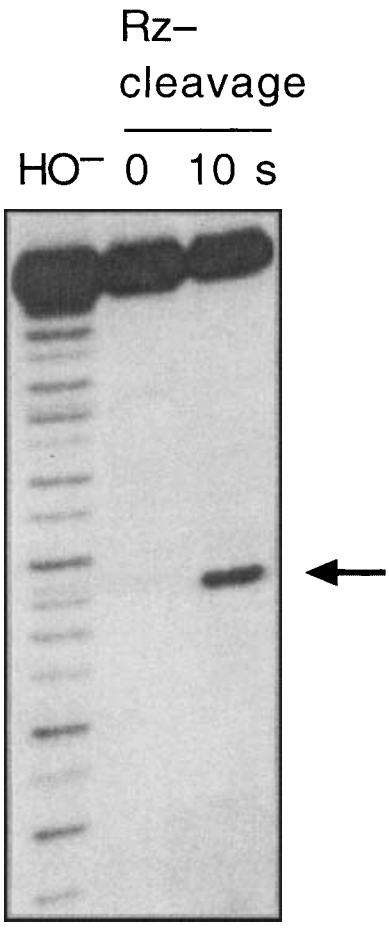
The Hammerhead Ribozyme







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Folding and Activity of the Hammerhead Ribozyme

Christian Hammann and David M. J. Lilley*[a]

The hammerhead is the smallest of the nucleolytic ribozymes, that undergo backbone cleavage by a transesterification reaction in the presence of magnesium ions. The RNA is induced to fold into its active conformation by the binding of metal ions in two stages. These generate domain 2, the scaffold on which the ribozyme is built, and domain 1, the active centre of the ribozyme. Further local structural rearrangement during the activation of the ribozyme is suggested by a number of crystal structures. The 10^5-fold rate enhancement is probably brought about by a combination of metal-ion participation and stereochemical factors in the environment of the folded RNA structure.

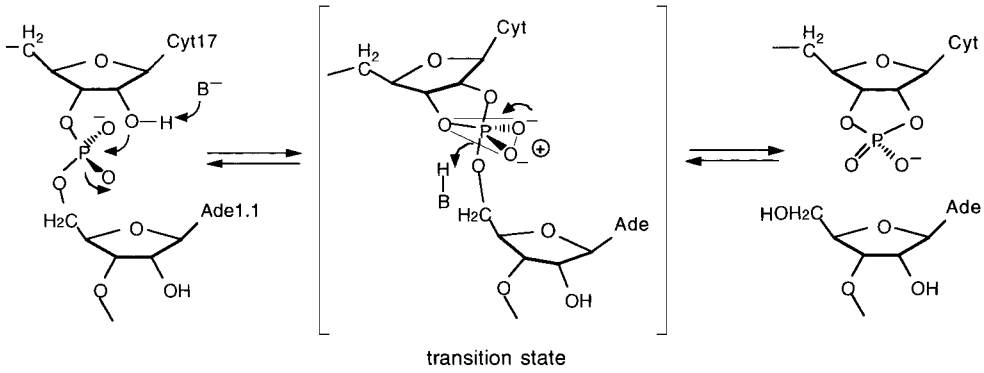
KEYWORDS:

catalysis · metal ions · ribozymes · RNA folding · thermodynamics

1. RNA Catalysis and the Nucleolytic Ribozymes

Ribozymes are RNA molecules that catalyse chemical reactions. The evolution of life on this planet may have passed through a stage (the "RNA world") during which RNA played simultaneous catalytic and informational roles, and perhaps the peptidyl transferase activity of the ribosome is a kind of molecular fossil from this period.[1] Whether or not that is true, RNA catalysis plays an important role in the cell, bringing about what is arguably the most important reaction in contemporary biology, that is, the synthesis of proteins. It is also highly probable that the splicing of mRNA will ultimately turn out to be RNA-catalysed. Thus, RNA catalysis is important from several different perspectives. Potentially it can also teach us a great deal about biocatalysis in general, since the ribozymes appear to be nature's minimalist catalytic macromolecules.

The nucleolytic ribozymes are a group of relatively small ($\leq$150 nucleotides) RNA species that carry out a site-specific cleavage of their own phosphodiester backbone by means of a transesterification reaction; they require only metal ions to achieve this.[2] There are four natural members of this class. These are the hammerhead,[3, 4] hairpin,[5, 6] hepatitis delta virus (HDV)[7] and Varkud satellite (VS)[8] ribozymes. Each has a very distinct sequence and structure, but they bring about essentially the same reaction. This is the accelerated attack of the 2′-oxygen atom on the 3′-phosphate group with expulsion of a 5′-oxygen and formation of a 2′,3′ cyclic phosphate.[9] In the course of the reaction the chirality of the phosphorus becomes inverted,[10–12] a fact that strongly suggesting an S_N2 reaction mechanism (Scheme 1). Some of these ribozymes can also bring about the reverse reaction,[5, 13] a ligation in which the 5′-oxygen atom attacks the phosphorus of the cyclic phosphate group; this is particularly efficient in the case of the hairpin ribozyme for example.[14] In addition to these natural ribozymes, the same reaction has been found to be catalysed in tRNA in the presence of lead ions,[15] and in selected RNA[16] and DNA species.[17]



Scheme 1. *The probable cleavage mechanism of the hammerhead ribozyme, and potential catalytic strategies. All available evidence points towards an S_N2 mechanism, whereby the 2′-oxygen atom attacks the 3′-phosphorus group with expulsion of the 5′-oxygen atom proceeding via the trigonal bipyramidal transition state. The reaction could be accelerated by removal of the proton from the 2′-oxygen atom by a base (shown as B^-) and protonation of the departing oxyanion (by B–H). The doubly charged transition state could be stabilised by juxtaposition of a positive charge. Lastly, the geometry of the in-line trajectory could be facilitated by local RNA stereochemistry.*

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While the nucleolytic ribozymes generally cleave themselves in cis (intramolecularly) in their natural situation, they can all readily be converted into species that act in trans (intermolecularly), analogous to an enzyme acting upon its substrate. These ribozymes typically cleave their substrates at rates in the region of $1\,min^{-1}$. While this is relatively slow compared to protein enzymes, it represents an acceleration of $10^5 - 10^6$ over uncatalysed background cleavage.[18, 19]

2. The Hammerhead Ribozyme

The hammerhead ribozyme was discovered in plant virus satellite RNA, where it acts as a site-specific self-cleaving unit in the processing of single-stranded RNA transcripts arising from rolling circle replication.[3, 4] However, it is not restricted to the plant kingdom, as the transcript of the satellite DNA in the newt is also cleaved by a hammerhead ribozyme.[20] Variant hammerhead ribozymes with altered specificity have also been obtained in selection experiments.[21]

The hammerhead ribozyme can be regarded as an elaborated three-way junction, that is, a $HS_1HS_7HS_3$[22] junction (Figure 1). Most of the essential nucleotides and functional groups are contained within the formally unpaired bases of the junction,[23–33] and thus we might expect that the folding of this junction would create the local environment in which catalysis can proceed.

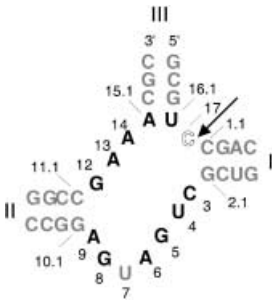

Figure 1. *The sequence of the hammerhead ribozyme. The hammerhead ribozyme comprises three helical stems, I, II and III, with a central core of formally unpaired nucleotides. The essential nucleotides are shown in black, with the nucleotide at the cleavage site (C17) highlighted in open text. The remaining sequences can be changed without severe loss of activity and will vary in different studies. Cleavage occurs at the position indicated by the large arrow. The conventional numbering of the nucleotides[103] is shown.*

3. Folding of Branched Nucleic Acids

Helical junctions abound in RNA molecules, where it is clear that they play major architectural roles. Many ribozymes contain junctions. The hairpin ribozyme for example is organised around a perfect (4H) four-way junction without which the folding is extremely inefficient,[34–36] while the VS ribozyme is constructed from the fusion of two three-way junctions.[37] Quite a lot is known about the folding of branched nucleic acids, in both DNA and RNA.[38] Two general principles can be established:

- There is a strong tendency to undergo pairwise, coaxial stacking of helical arms. This is very clearly seen in the four-way DNA junction,[39–41] but also occurs readily in RNA, as exemplified first by tRNA.[42, 43] Four-way junctions can stack in one of two equivalent ways, that depend on the choice of stacking partner. In the case of the hairpin ribozyme, the helical arms stack in such a way as to permit intimate association between the two loop-bearing arms.[34, 44] In the case of the three-way junction, this is rather more complicated. Clearly only two arms can stack coaxially, leaving the third unstacked, and there are two stereochemically non-equivalent conformers. In general three-way junctions are imperfect, with formally unpaired nucleotides that have a strong influence on the folding; in fact for DNA the perfect 3H junction cannot fold.[45, 46]
- Electrostatics are very important in the folding of helical junctions, and in general these junctions cannot fold in the absence of metal ions. Both diffuse and site-specific binding may be important in achieving the correct folding.

The hammerhead ribozyme illustrates both of these folding principles.

Christian Hammann, born in 1969, studied chemistry and biochemistry at the University of Regensburg, and at King's College, London. After obtaining his diploma thesis under Robert Huber at the Max-Planck Institute for Biochemistry in Munich, he joined Martin Tabler's group at the Institute for Molecular Biology and Biotechnology in Heraklion, Crete, Greece, where he worked on the characterisation of long-armed hammerhead ribozymes. In 1999 he received his PhD from the University of Regensburg, Germany. Since then he has worked with David Lilley on the folding and catalysis of small nucleolytic ribozymes. For this work he was awarded the Karl-Lohmann-Preis of the Gesellschaft für Biochemie und Molekularbiologie (GBM) in 2001.

David M. J. Lilley is a professor at the University of Dundee, where he is Director of the Cancer Research UK Nucleic Acid Structure Research Group. He originally trained as a chemist and obtained his PhD in theoretical chemistry. But since that time he has worked at the chemistry – biology interface in the general area of nucleic acids and particularly branched species in DNA and RNA. He has developed a number of novel approaches, including FRET spectroscopy, for the analysis of these species in solution. He is probably best known for elucidating the structure of the four-way (Holliday) DNA junction, and he has also made many studies of the interaction of these structures with enzymes. His studies of branched RNA have taken him into the area of RNA catalysis, and he has made significant contributions to research into the folding and catalysis in the hammerhead, hairpin and VS ribozymes. David Lilley has also published extensively in the areas of guanine tetraplexes and the geometry and genetics of DNA supercoiling. Professor Lilley has just been elected a Fellow of the Royal Society.

4. The Structure of the Hammerhead Ribozyme in the Crystal

The structure of the hammerhead ribozyme was solved by crystallography independently in the laboratories of McKay[47] and Scott.[48] The two structures were closely similar, despite differences in strand connectivity and chemical composition of the substrate strand (that is, RNA or DNA). The ribozyme forms a Y-shaped structure, in which there is coaxial alignment between stems II and III, and with stem I directed in the same quadrant as stem II (Figure 2 A). The global shape of the structure in the crystal was in good agreement with solution structures from fluorescence resonance energy transfer (FRET) spectroscopy,[49, 50] comparative gel electrophoresis[51] and transient electric birefringence.[52]

The structure of the ribozyme in the crystal[47, 48] is organised around two structural features. Domain 2 is formed by base mispairing between the oligopurine section lying between stems II and III and the U7G8A9 sequence at the 3′ end of the long section connecting stems I and II (Figure 2 B). Two G·A mismatched basepairs are formed, together with a noncanonical A–U pair connected by a single hydrogen bond. The terminal adenine (A15.1) of helix II, A14 and A13 form a continuous stack. A13 forms a cross-strand stacking with A9 (as do their pairing partners G8 and G12), which in turn is stacked onto the terminal guanine (G10.1) of helix III. Thus, there is unbroken stack of purine bases running through domain 2, thereby mediating the coaxial alignment between helices II and III. The strand containing the G12A13A14 sequence is a typical continuous strand of a junction, that runs smoothly from helix II through to helix III.

Domain 1 is formed from the C3U4G5A6 sequence at the 5′ end of the long single-stranded section (Figure 2 C). This forms a uridine turn, as first seen in the anticodon loop of tRNAPhe.[42, 53] The UGA sequence conforms to the UNR motif that forms a sharp turn in the trajectory of the backbone between U4 and G5, so that the base of A6 becomes almost perfectly stacked with that of G5, on the same side as U4. This loop of RNA is close to the cleaved 3′-phosphate group of C17 and was therefore presumed

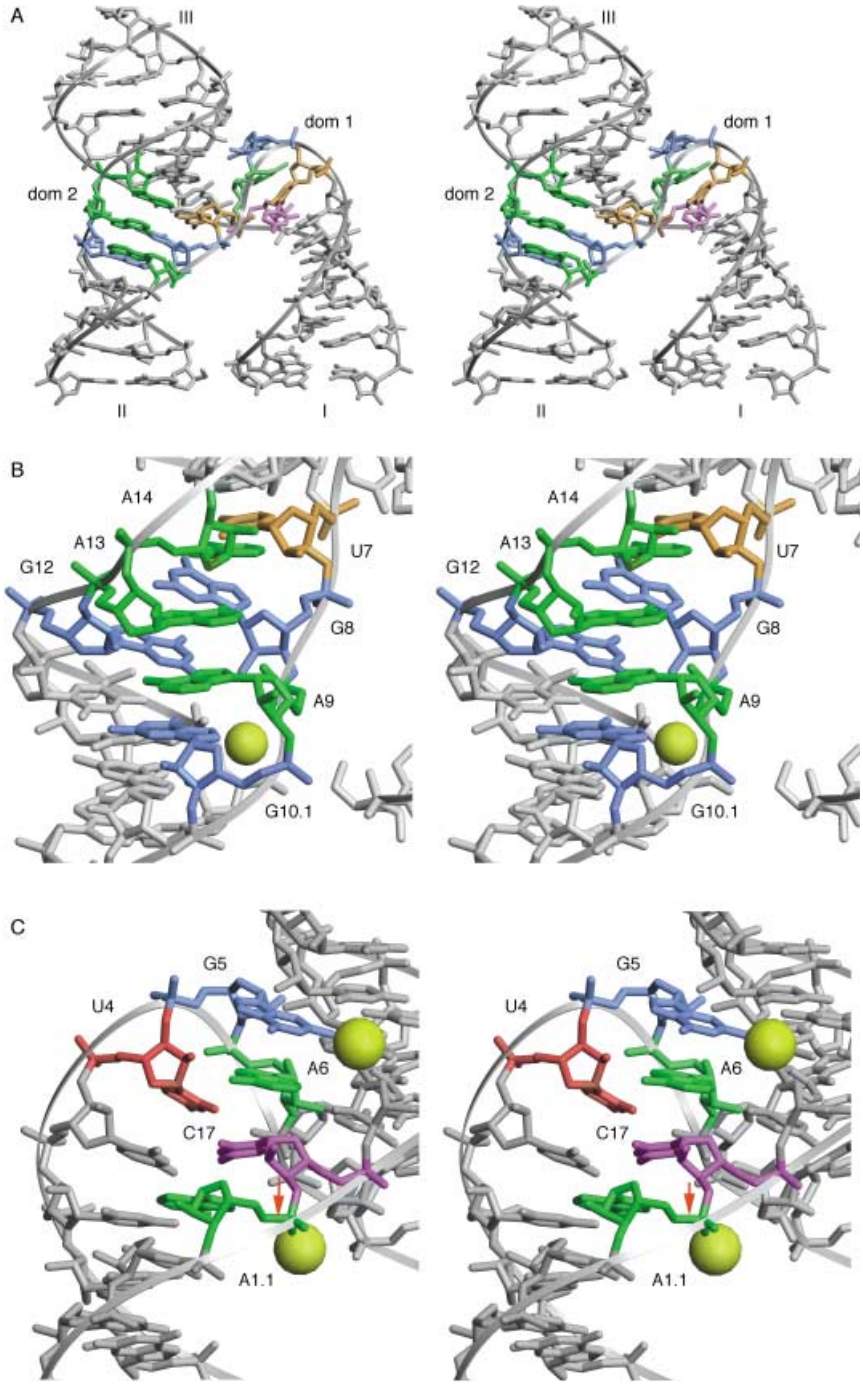

Figure 2. *The structure of the hammerhead ribozyme in the crystal. A) Parallel-eye stereoscopic view of the structure of the complete ribozyme. The view is from the minor groove side of the junction, with the three helices identified. The ribbon indicates the path of the backbone. Two regions of the ribozyme have been highlighted in colour (guanine = blue, adenine = green, cytosine = magenta and uracil = orange). Domain 2 mediates the coaxial alignment of helices II and III, while domain 1 is the uridine turn that surrounds C17 at the cleavage site. This image was generated from the coordinate file URX057, deposited with the PDB by Scott and co-workers.[66] It comprises an all-RNA ribozyme, prior to soaking by divalent metal ions. B) Parallel-eye stereoscopic view focussed on domain 2, viewed from the same side as in (A), showing the two consecutive G·A mismatches and the continuous set of purine stacking interactions. The yellow sphere shows a Mn^{2+} ion bound to the A9 phosphate group and the nitrogen at position 7 of G10.1. This image was generated from the PDB coordinate file URX058. C) Parallel-eye stereoscopic view focussed on domain 1. This is viewed from the opposite side to (A), that is, from the major groove side of the junction. The U4G5A6 turn is highlighted, along with the C17A1.1 of the cleaved strand. The scissile bond is arrowed. Two Mg^{2+} ions are shown in this image as yellow spheres, bound to the scissile phosphate group and G5. Note that the local structure around the cleavage site is rearranged relative to that shown in (A); see text. This image was generated from the PDB coordinate file URX059.*

to be the catalytic core of the ribozyme. The phosphodiester linkage between A6 and U7 can be regarded as the crossover point of the three-way junction, and is the connection between domains 1 and 2.

5. Ion-Induced Folding of the Hammerhead Ribozyme in Solution

The hammerhead ribozyme undergoes a well-defined process of folding induced by the binding of Mg^{2+} and other metal ions to achieve the active conformation observed in the crystal. In the absence of added metal ions the hammerhead is extended, with no coaxial stacking of helical arms.[50, 51, 54] Upon titration of Mg^{2+} ions, the hammerhead exhibits a two-stage folding process[50, 51] (Figure 3). With Mg^{2+} ions in the concentration range of 0 – 500 μM, helices II and III undergo coaxial stacking. Examination of the structure seen in the crystal suggested that this might correspond to the formation of domain 2, consistent with the observation that the mutations A14G (lying in the oligopurine sequence between stems II and III) and G8U (on the opposite strand) completely block this stage of the folding process. Analysis of titrations based on FRET measurements indicated that the transition is induced by the noncooperative binding of Mg^{2+} ions,[50] with an apparent association constant in the region of 10 000 M^{-1}. Raising the concentration of Mg^{2+} ions further, over the range 0.5 – 20 mM, induced a second change in conformation, in which helix I changed relative position from being in the same quadrant as helix III to lying quite close to helix II. No further structural changes were observed at higher ionic concentrations, and the final global structure attained was clearly in good agreement with that in the crystal. We proposed that the second transition reflected the formation of the uridine turn by the C3U4G5A6 sequence. The transition was found to be perturbed by changes in this sequence, such as G5C and deoxy-G5.[55, 56] The latter effect can be explained by a disruption of the hydrogen bond observed in the crystal between the 2′-hydroxy groups of G5 and C15.2 in helix III;[47] this change also leads to a severe lowering of catalytic activity.[26] Analysis by FRET spectroscopy indicated that this transition was also induced by the noncooperative binding of Mg^{2+} ions, but the calculated apparent association constant was an order of magnitude lower than that for the first transition. The range of Mg^{2+} ion concentration over which the second transition occurs is very similar to that over which catalytic activity is acquired,[57] suggesting that the final structure is at least close to a catalytically competent structure.

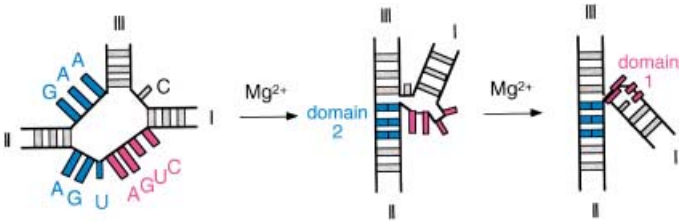

Figure 3. *The folding of the hammerhead ribozyme is a two-stage process induced by the binding of metal ions. A schematic to illustrate the folding process for the hammerhead ribozyme. This occurs as two structural transitions, each resulting from the noncooperative binding of a divalent metal ion. Domain 2 is formed in the first stage, induced by the binding of a metal ion, with a* $K_A \approx 10{,}000\ M^{-1}$*. At this intermediate stage the ribozyme is not active. The second stage is induced by the noncooperative binding of a second divalent metal ion, with a* $K_A \approx 1{,}000\ M^{-1}$*, and corresponds to the formation of domain 1. At this point the structure is essentially that observed in the crystal.*

6. Folding in Solution Defined at the Local Level

The two-stage ion-induced folding scheme illustrated in Figure 3 was largely inferred from measurements of the relative dispositions of helical arms, either by comparative gel electrophoresis or FRET spectroscopy. This "outside-in" approach cannot provide direct information on which nucleotides are participating at given stages of the folding process, and for this we require information coming from the core itself. Thus, we want to slip a "spy on the inside" to provide this new perspective, by the insertion of reporter groups at particular locations in the ribozyme core.

Two such experimental approaches have been taken, using 2-aminopurine (2-AP) fluorescence[58] and ^{19}F nuclear magnetic resonance (NMR) spectroscopy.[59] Modified bases were introduced by chemical synthesis at selected positions, and their spectroscopic properties studied as a function of Mg^{2+} ion concentration. It should be noted that Mg^{2+} ion induced changes in an observable do not necessarily indicate direct metal ion coordination at the modified position. The modification rather serves as a reporter that indicates ion-induced conformational changes within the hammerhead.

Changes in 2-AP fluorescence were observed, both for association of a substrate and a ribozyme strand in a trans ribozyme, and upon titration with Mg^{2+} ions in cis ribozymes with the fluorescent base incorporated at various sites.[58] From the latter, Mg^{2+} ion binding constants could be determined that ranged from 7600 – 12 M^{-1} and indicated multiple binding sites with varying affinities for Mg^{2+} ions. Changes in 2-AP fluorescence could be interpreted as single binding processes for ribozymes that contained 2-AP in the core of cleavage-incompetent, but otherwise unchanged, ribozymes. An additional binding event was observed for an A14G variant, that had been shown to be folding-incompetent,[51] and for a hammerhead with 2-AP in the external loop 3. In a second set of experiments, 2-AP incorporated at various sites was used to study the dynamics of the hammerhead ribozyme by measurement of fluorescence-detected temperature jump relaxation.[60] The observed relaxation time constants ranged from a few microseconds to 200 ms, with the folding-incompetent A14G variant exhibiting a particularly slow relaxation process. In most cases, the data indicated at least three relaxation processes in the background of a constant Mg^{2+} ion concentration on the pre-steady-state timescale, which however could not be attributed to particular structural changes. Despite this, the data demonstrated the existence of multiple relaxation processes and provided evidence for local relaxation modes.

More recently, we have studied the metal-ion-induced folding of the hammerhead ribozyme by selectively incorporating 5-fluorouridine (5-FU) at positions 4 and 7 in the hammerhead core for use in ^{19}F NMR experiments.[59] The choice of these positions was largely guided by the two-stage folding pathway as described in Figure 3, as U4 lies within domain 1 and U7 is within domain 2 and adjacent to domain 1 (Figure 4A). From changes in the chemical shift and linewidths of the ^{19}F resonances upon titration with Mg^{2+} ions, we showed that U4 and U7 both participate in the formation of domain 1. This was induced by the noncooperative binding of Mg^{2+} ions with association constants of about 500 M^{-1} (Figure 4B). Further, U7 (but not U4) underwent an additional conformational change in the submillimolar Mg^{2+} ion concentration range, in which domain 2 is formed (Figure 4C). The NMR spectroscopy approach proved especially useful as each titration point could be studied by two different properties, that is, chemical shift and linewidth. These results were fully consistent with the previously proposed folding scheme (Figure 3). U4 is sensitive only to the second (higher Mg^{2+} ion concentration) transition, since this is the folding of domain 1. It is completely insensitive to the formation of domain 2 occurring at the lower Mg^{2+} ion concentration. By contrast U7 sits at the interface between the two domains, and is therefore sensitive to the folding of both. Thus, the NMR spectroscopy results support the original proposal for the two-stage folding process. We see that the initial (that is, low Mg^{2+} ion concentration) transition corresponds to the formation of domain 2, the scaffold on which the complete structure is ultimately built. Domain 1, the catalytic core of the ribozyme, becomes folded in the second transition.

During the initial transition, the ^{19}F resonance linewidth of U7 significantly broadened, and then subsequently sharpened towards the end of the transition (Figure 4C). This suggests that it is in an intermediate exchange regime, and consequently we were able to calculate that the formation of domain 1 occurs on a timescale in the range of one millisecond.

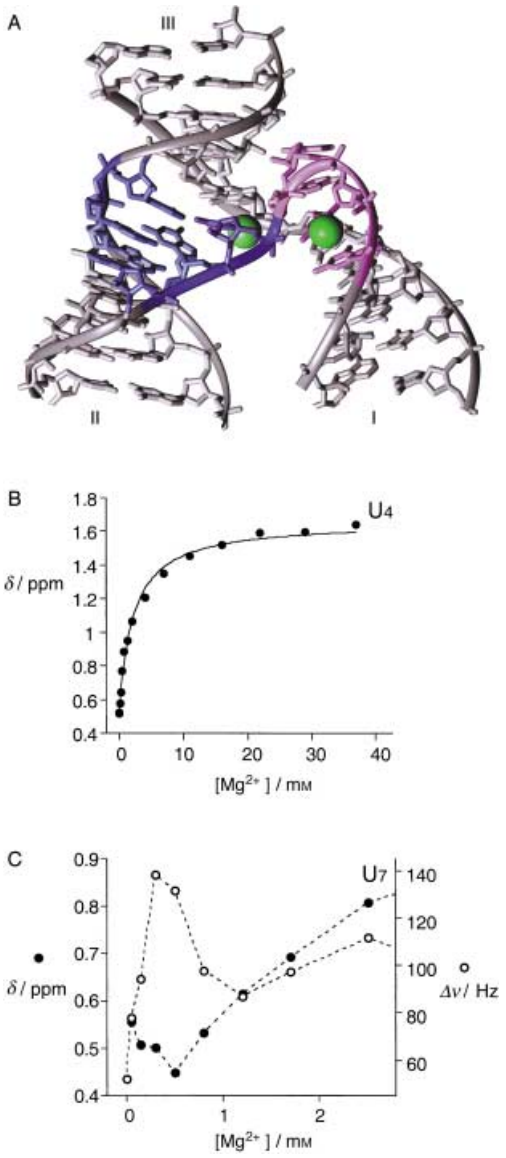

Figure 4. *The folding of the hammerhead ribozyme analysed at the local level by means of ^{19}F NMR spectroscopy. A cloverleaf form of the ribozyme was made by chemical synthesis, containing a single 5-fluorouracil (FU) at either the 7 or 4 positions. It was anticipated that the ^{19}F nucleus would experience an altered magnetic environment upon changes in the local conformation induced by the binding of metal ions. A) The structure of the hammerhead ribozyme with the positions of the fluorine atoms used in the NMR study indicated in green. FU4 is centrally located in domain 1 (highlighted in magenta in this image), while FU7 is located in domain 2 (highlighted in blue) close to the boundary with domain 1. B) Plot of the chemical shift (ppm; filled circles) of the FU4 ^{19}F resonance as a function of added Mg^{2+} concentration in 10 mM tris(hydroxymethyl)aminomethane·HCl (Tris·HCl; pH 7.5) and 10 mM NaCl. The data have been fitted to a two-state model in which the transition is induced by the binding of a single magnesium ion (line). C) Plot of the chemical shift (ppm, filled circles) and linewidth (Hz, open circles) of the FU7 ^{19}F resonance as a function of added Mg^{2+} concentration in the presence of 10 mM Tris·HCl (pH 7.5) and 10 mM NaCl. There is a small upfield shift in the 0–500 μM Mg^{2+} ion range. In the same range the linewidth increases and then narrows.*

7. Metal-Ion Binding

The folding of the hammerhead ribozyme is induced by the addition of divalent metal ions. This suggests that in the absence of the ions folding is prevented by electrostatic repulsion, and the net free energy of folding only becomes negative when this is reduced by the binding of counterions. We would envisage that the folding creates electronegative clefts and that these must be occupied by cations to allow the folding to occur. Binding into such sites might be formally diffuse and essentially Coulombic, whereby the metal ion retains its inner coordination sphere, and it is in fast exchange with ions in the bulk solvent. Alternatively, if atoms from the RNA provide inner-sphere ligands the metal ion may be held in a more static manner. It is not always easy to distinguish these two types of binding. Ions may be held by outer sphere interactions, yet despite rapid exchange with the solvent the binding site may exhibit high occupancy. Both types of binding are required for the complete folding of the hairpin ribozyme.[61] Metal-ion binding to the hammerhead ribozyme has been studied in solution, by using chemically active metal probes such as UO_2^{2+}[51] and Tb^{III},[62] by NMR spectroscopy[63, 64] and by EPR spectroscopy.[65]

A number of ion binding sites have been identified in the hammerhead ribozyme by crystallography.[47, 48, 66] Several of

these are in the helical arms, but three lie in particularly interesting locations. Metal ions have been observed bound at the phosphate group of A9 in a number of structures, making inner-sphere contacts with the *pro*-R oxygen atom of the A9 phosphate group and the nitrogen atom at position 7 of G10.1 (Figure 2B). This would be a strong candidate for the metal ion that is responsible for the first stage of folding, that results in the formation of domain 2. We have observed that phosphorothioate substitution of this phosphate leads to a significant impairment of folding by Mg^{2+} ions, and this can be restored by replacement with Mn^{2+} ions (G. S. Bassi and D.M.J.L., unpublished data). ^{31}P NMR spectroscopy has been used to demonstrate cadmium ion binding to an A9 phosphorothioate.[64] A second general area of binding has been found close to G5 in the uridine turn of domain 1 (Figure 2C). This was first suggested by uranyl-induced photocleavage experiments,[51] and it was later shown that this site exists only in the fully folded structure.[56] By using crystallography, an Mn^{2+} ion has been observed bound in this region,[48] while a Tb^{III} ion was found to be bound to the face of the base of G5.[67] It is possible that metal ion binding in this region could be required for formation of domain 1, but at the present time this cannot be concluded with any certainty. Lastly, metal ions have been found close to the scissile bond in the hammerhead,[48, 66] where they would be well placed to participate in the chemistry of cleavage. For example, a Mg^{2+} ion binding site was deduced in flash-frozen crystals of hammerhead ribozyme at pH 8.5, whereby the metal would be bound to the *pro*-R oxygen atom of the scissile phosphate group[66] (Figure 2C).

8. Energetics of Hammerhead Folding

We have studied the thermodynamics of the hammerhead ribozyme folding using isothermal titration calorimetry.[68] $MgCl_2$ was titrated into a concentrated solution of a cleavage-incompetent, but otherwise unchanged, cloverleaf hammerhead ribozyme, and the heat evolved or absorbed was compared to that of sequence variants that either undergo only the first (G5C) or neither (A14G) of the folding events shown in Figure 3. The natural sequence ribozyme exhibited exothermic binding of Mg^{2+} ions (Figure 5A), and the integrated heat data were analysed in terms of sequential interactions at two sites, with association constants $K_A = 480\,M^{-1}$ and $2840\,M^{-1}$. The two sequence variants gave very different isothermal titration curves, thereby confirming their highly perturbed folding. The A14G variant (that does not undergo ion-induced folding), underwent endothermic binding, while the dG5 variant (that can undergo only the first folding transition) yielded a complex titration curve. However, the data for these two variants each fit the sequential binding of Mg^{2+} ions at two sites. This is perhaps surprising, as one might have expected that the failure to undergo a particular stage of folding might result in the loss of the associated ion binding site. The observation of two ion binding sites in the A14G sequence variant is supported by the 2-AP fluorescence data for this RNA.[58] The metal ion affinities for the three RNA species in the calorimetric study were all in the region of $10^3\,M^{-1}$, corresponding to free energies of $\Delta G^\circ = -3.5$ to -4 kcal mol^{-1}.

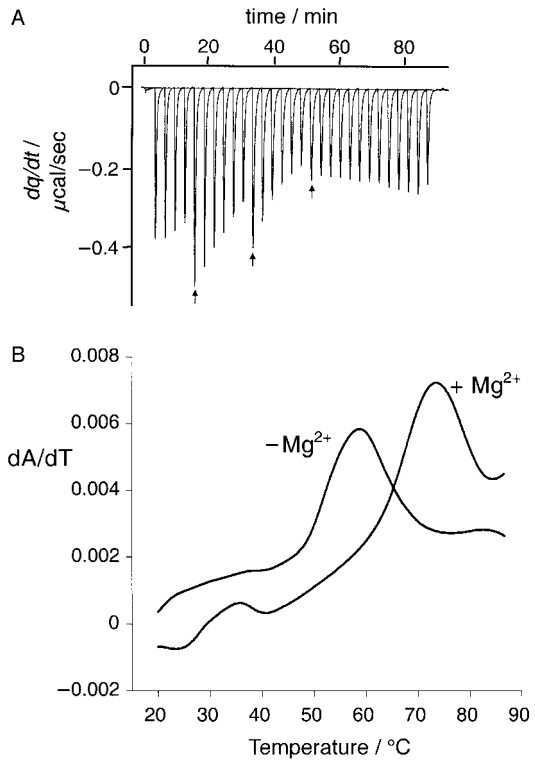

Figure 5. *The thermodynamics of the folding process of the hammerhead ribozyme. A) Isothermal titration calorimetry of the interaction of Mg^{2+} ions with the natural sequence hammerhead ribozyme.[68] Plot of heat evolved as a $MgCl_2$ solution is titrated into a solution of the hammerhead ribozyme in the form of a cloverleaf structure in 50 mM Tris·HCl (pH 8.0) and 100 mM NaCl at 283 K. The apparent discontinuities in the heat profile are due to changes in injection volumes, at the positions indicated by arrows. These data are a good fit to a sequential two-ion binding model. B) Thermally-induced melting of the hammerhead ribozyme followed by the absorption of light at 258 nm. Plot of the first derivative of the absorption of UV light with respect to temperature (dA/dT) as a function of temperature for a cloverleaf form of the hammerhead ribozyme in 25 mM Na cacodylate (pH 6.5) and 25 mM NaCl, with and without addition of 10 mM $MgCl_2$.*

Thus, the enthalpic and entropic contributions compensate, to result in similar binding affinities for the RNA species tested.

Based on calorimetric data measured at two temperatures, the heat capacities (ΔC_p) for the two ion binding events for the natural sequence were found to be very small. Compared to this, some ion binding events in the sequence variants showed considerable positive or negative changes in ΔC_p. A recent study employing CD spectroscopy revealed that the unfolding of the secondary structure of the hammerhead ribozyme could occur both by hot and cold denaturation.[69] This was the first direct observation of cold denaturation in nucleic acids, a phenomenon which is caused by differences in the heat capacities of folded and unfolded states. Based on CD spectra recorded at temperatures ranging from 250–350 K, a ΔC_p value of ≥ 43 cal K^{-1} (mol bp)$^{-1}$ was determined for the unfolding of the hammerhead's secondary structure in 500 mM monovalent ions. For the temperature range between 280 and 320 K, however, there was only a small change in elipticity. As the hammerhead ribozyme is active to some extent (but not fully) under these

ionic conditions,[70] it is possible that these small changes in elipticity correspond to changes in tertiary interactions.

This interpretation is supported by the observation of absorbance changes in UV melting experiments in a similar temperature range. We have analysed the thermally induced melting of a cloverleaf hammerhead species in the presence of 25 mM NaCl, with and without an additional 10 mM Mg^{2+} ions (Figure 5B; C.H. and D.M.J.L, unpublished data). They exhibit a large amplitude high-temperature transition at 59 ($-Mg^{2+}$) and 73 °C ($+Mg^{2+}$), which must correspond to the melting of the basepairing in the helical arms. However, in the presence of Mg^{2+} ions there is a smaller amplitude transition occurring at lower temperature, around 37 °C. This is also observed in the G5U variant, but is not present for the G8U variant. This is therefore likely to arise from the melting of the domain 2 scaffold, and thus the tertiary structure of the wild-type hammerhead ribozyme.

9. Are Further Conformational Changes Required for Activity in the Hammerhead Ribozyme?

There is good agreement between the global shape of the hammerhead ribozyme in solution found by a range of techniques including comparative gel electrophoresis,[51] FRET spectroscopy[49, 50] and transient electric birefringence.[52] This structure also corresponds well with that observed in the crystal, despite the use of different constructs with either 2′-deoxy[47] or 2′-*O*-methyl[48] substitution at the cleavage site. The hammerhead was the first of the nucleolytic ribozymes to yield to crystallography, and many expected that the geometry at the cleavage site would be predisposed to S_N2 attack, perhaps with close to colinearity of the attacking 2′-oxygen, the 3′-phosphorus and the departing 5′-oxygen atoms. It was recently found, for example, that the geometry at the corresponding position of the hairpin ribozyme is within 40° of colinearity.[44] However, the first structures for the hammerhead ribozyme showed that the geometry of the backbone at the cleavage site was close to that in an A-form helix, and differed maximally from the required in-line trajectory; that is, the angle relating the three participating atoms was not 180° but 90°.[47, 48] This suggests that the structure originally observed was held in a ground-state conformation, that would therefore be required to undergo further changes to catalyse the transesterification reaction. We might therefore wonder how extensive a rearrangement of the structure would be required. It should be noted that the ribozyme is significantly active in the crystal when metal ions are diffused into the lattice;[71, 72] this suggests that a gross conformational change involving the reorientation of helices cannot be essential, and a crystal structure of the product of the reaction has the same global fold.[73]

Since the original crystal structures, Scott and co-workers have obtained a number of structures in which the geometry at the cleavage site is closer to that expected for an in-line attack. By using rapid freezing of an all-RNA hammerhead ribozyme, Scott et al.[66] observed a 3 Å conformational change around the active centre. This moved the substrate strand relative to the catalytic pocket and created new metal-ion binding sites. However, a more substantial rearrangement was observed by using a kinetically impaired substrate strand that allowed intermediates to accumulate in the crystal. A methyl substitution at the C5′ position of A1.1, which is adjacent to the cleaved phosphate group, reduced the rate of cleavage by 300-fold, thus acting as a kinetic bottleneck. Crystals were soaked in cobalt(II) ions for 2.5 h, before freezing and crystallographic study.[71] The resulting structure was found to be substantially altered from the ground state, with a coordinated 7.8 Å movement at the active centre. The base and ribose of C17 were rotated by 60°, flipping the 2′-hydroxy group towards the scissile phosphate. The net result was a local structure that was much closer to the expected in-line geometry. Recently Murray et al.[72] have shown that the cleavage rate in the crystal is very pH-dependent and have suggested that the conformational change is rate limiting and governed by the ionisation of a 2′-proton.

It should be noted that the global structure of these different forms is not substantially altered from that observed in the ground-state structure, and the changes are largely limited to the active centre. The structural scaffold formed by domain 2 is essentially unaltered, and thus the importance of the initial folding of this feature is undiminished. However, a larger scale structural change during activation has been proposed. Based on a very careful measurement of phosphorothioate effects at multiple sites, and their rescue by using soft, thiophilic metal ions, Herschlag and co-workers[74] have argued that in the transition state a single metal ion becomes simultaneously bound to the phosphate group at A9 (that is, within the domain 2 scaffold) and the scissile phosphate group. In the normal structure these phosphate moieties are separated by a distance of around 20 Å, and thus it would require a very substantial structural alteration to bring them close enough to coordinate the same metal ion simultaneously. By using their crystallographic structures as a starting point, Murray and Scott[75] tried to model such a conformation, but concluded that this would require a disruption not only of the core but also the helices. This would be difficult to reconcile with the cleavage activity measured in the crystal. Moreover, when a cross-link was placed between helices I and II, thereby constraining major excursion from the global structure, the ribozyme remained active.[76] Interestingly, however, it has been shown that such a cross-link can result in a marked elevation in the rate of ligation by the hammerhead ribozyme.[77]

10. Origins of Catalysis in the Hammerhead Ribozyme

Ion-induced folding of the hammerhead ribozyme is a prerequisite for catalytic activity, and there is a good correspondence between the concentrations required to achieve folding and those giving maximal rates of cleavage. Folding creates the local environment in which the transesterification reaction is accelerated by at least 10^5 fold at the phosphate group of C17. Can we identify the catalytic strategy employed by the ribozyme? Returning to the mechanism of the reaction shown in Scheme 1, we can suggest a number of points of potential intervention. First, a hydroxy group is a poor nucleophile, and the rate could

potentially be greatly increased by using a general base to remove the proton from the attacking 2′-hydroxy group.[19] Second, departure of the 5′-oxyanion may well be rate limiting,[78, 79] and thus its protonation by a general acid could be important. Third, the pentacoordinate oxyphosphorane transition state carries two negative charges, and so juxtaposition of a positive charge could reduce the activation energy. Last, the correct local geometry of the attacking nucleophile, phosphorus and leaving group will be required to develop the in-line trajectory for the S_N2 reaction. There are three potential players that could mediate the catalysis. First, metal ions could play a direct role in the chemistry of cleavage, over and above their established importance in ensuring folding into the required conformation. Water present in the first coordination sphere could participate in general acid/base catalysis,[80] or, alternatively, either the attacking nucleophile or leaving groups could be activated by direct coordination. A metal cation could also stabilise the transition state electrostatically.[57] Second, the nucleobases could potentially play a significant role in general acid/base catalysis. The best candidates are the nitrogen atoms at position 3 of cytosine or position 1 of adenine, although guanine might also participate. To function catalytically at near to neutral pH requires a significant alteration in pK_A, but this is easily possible in the environment of a highly charged nucleic acid, and altered pK_A values of adenine have been measured by NMR spectroscopy in the leadzyme.[81] Lastly, the folding of the RNA can lead to local structural distortion that facilitates the trajectory into the in-line transition state.

The best evidence for nucleobase catalysis has been obtained for the HDV ribozyme.[82, 83] C75 is poised adjacent to the scissile bond in the crystal structure,[84] and mutation of this nucleotide is very deleterious. However, activity in a C75U mutant can be significantly restored by addition of exogenous imidazole in the solution.[83] Perhaps surprisingly, recent measurement by NMR spectroscopy showed that the pK_A of C75 was not significantly elevated towards neutrality,[85] but it is possible that this may only occur transiently during the activation of the ribozyme.

There is also suggestive evidence for a similar role for G8 in the hairpin ribozyme;[86, 87] with the advent of the crystal structure, this base was found to be located immediately adjacent to the scissile phosphate group, indeed it is hydrogen bonded to it.[44] In the VS ribozyme there is circumstantial (though not yet conclusive) evidence pointing towards a role for the nucleobase of A756.[88] However, despite virtually saturation coverage of the hammerhead ribozyme by changes of base and functional groups, no convincing case has been established for nucleobase involvement in catalysis. Furthermore, no good candidate has emerged from the crystallographic analysis.

There have been a number of studies indicating a direct role for metal-ion catalysis in the hammerhead ribozyme. When the nonbridging oxygen atoms at the phosphate group adjacent to the scissile bond were individually substituted with sulphur it was found that the rate of cleavage was reduced by two orders of magnitude for the *pro*-R oxygen atom, and that cleavage activity could be restored if Mg^{2+} ions were replaced by the more thiophilic Mn^{2+} ion.[11, 57, 89] A metal ion was observed bound to the *pro*-R oxygen atom of the scissile phosphate group in flash-frozen crystals of hammerhead ribozyme at pH 8.5[66] as discussed above. The pH dependence of cleavage rate was interpreted in terms of a role for a metal hydroxide,[80] and suggested a model in which a metal ion bound at the *pro*-R oxygen atom abstracted the proton from the 2′-hydroxy moiety. Von Hippel and co-workers argued that this mechanism would fail to explain the dependence on pK_a, because of a cancellation of equilibria, and suggested that the pH dependence could be better explained in terms of the equilibrium concentration of metal hydroxide.[90] They proposed that the metal ion activates the 2′-oxygen atom by forming an inner-sphere association, thereby acting as a Lewis acid catalyst. A variety of two-metal-ion models have also been suggested, proposed originally for phosphoryl-transfer reactions in enzymes.[91] For example, Lott et al.[92] proposed that two-metal-ion binding events were required to explain a bell-shaped activity dependence when they titrated the hammerhead ribozyme with lanthanum ions in the presence of a constant background of Mg^{2+} ions.

Some doubt has been cast on a direct role for metal ions. The hammerhead ribozyme exhibits significant activity in the presence of high concentrations of monovalent metal ions, and even ammonium ions,[70] as the sole cations. Similar observations were also made for the hairpin and VS ribozymes, consistent with other reports that the hairpin ribozyme is active in the substitutionally inert hexammine cobalt(III) ions[93–95] and aminoglycoside antibiotics and polyamines.[96] Since the ammine ligands of the Co^{III} complex are substitutionally inert this result precludes a requirement for inner-sphere coordination or a role for coordinated water. Activity in high concentrations of monovalent metal ions need not exclude a role in catalysis, but it does alter our view. Such ions are very unlikely to be bound site-specifically, that is, directly coordinated to the active centre. They could nevertheless exhibit high occupancy close to the scissile bond, where they could play an important electrostatic role in the reaction.

The remaining factor is RNA conformation, arising from the requirement for an in-line attack of the 2′-oxygen atom. Soukup and Breaker[97] have defined an "in-line fitness" parameter that measures the angle of attack and the proximity of the attacking nucleophile in the base-catalysed breakage of the backbone at different positions in a number of RNA species. They obtain a degree of correlation between this parameter and cleavage rates over about three orders of magnitude. It is hard to obtain a good estimate of how much rate enhancement can be obtained from orientation factors, relative to the rate of cleavage of a flexible dinucleotide, but it is probably not worth more than a factor of 100. Recently Scott[98] has pointed out that alignment of the attacking and departing oxygen atoms is not enough in itself. He noted that in one of the crystal structures of the hammerhead, the geometry around the phosphate group at A9 is almost perfectly in-line, yet the rate of cleavage is very low. By noting the hydrogen-bonding pattern at the 2′-hydroxy group Scott deduced that the lone pairs of the oxygen atom are directed away from the vacant d orbitals of the phosphorus atom. Thus, despite the aligned atomic positions, the molecular orbital alignment is far from optimal. Interestingly, Sigurdsson and co-workers[99] have obtained cleavage at the A9 site of the

hammerhead in the presence of zinc ions. Evidently inappropriate orbital orientation can result in severely reduced cleavage activity despite apparently good stereochemical alignment. However, it is not clear that the argument can be reversed to conclude that large enhancements of cleavage rates can be achieved by favourable orbital alignment (when the reference state is a flexible dinucleotide), as has been discussed extensively for protein enzymes in the past.[100–102]

It is likely that the rate enhancement of around 10^5-fold observed in the hammerhead ribozyme, as with other nucleolytic ribozymes, arises as the sum of a number of factors that include metal ions (not necessarily bound site-specifically) and stereochemistry. But whatever the mechanism proves to be in detail, it can only function in the local environment provided by the correct folding of the RNA. Thus the metal-ion-induced folding is the essential first stage on the road to catalytic activity, and the hammerhead ribozyme provides a relatively uncomplicated system in which to study this.

11. Conclusion

The hammerhead ribozyme requires ion-induced folding into the correct structure for cleavage to proceed. There is excellent agreement between the global structure of the folded structure in the crystal and in solution. The ribozyme folds in two stages, each induced by the noncooperative binding of metal ions. In the first stage the structure of domain 2 occurs, possibly due to the binding of a divalent metal ion at the A9 phosphate group. This creates the scaffold on which the ribozyme is ultimately constructed. The second stage of folding results in the formation of the uridine turn, and creates the catalytic core of the ribozyme. This generates the local environment in which catalysis can proceed, probably due to a combination of metal ion participation and stereochemical factors. However, significant further local conformational changes at the cleavage site are required to occur prior to the cleavage step itself.

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