The Chirality of a Four-Way Helical Junction in RNA

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Helical junctions are commonly occurring elements in RNA structure. They play a key role in small autonomously folding RNA molecules. Four-way junctions in DNA and RNA exhibit a strong propensity to undergo pairwise coaxial stacking of helical arms[1] and a dependence of the structure on metal ions. A four-helix junction in DNA with no unpaired bases (4H)[2] adopts a right-handed, antiparallel stacked structure in the presence of Mg^{2+} ions in solution,[3,4] and in the crystal.[5,6] 4H RNA junctions tend to adopt an antiparallel structure in the presence of divalent metal ions[7,8] of unknown handedness. The hairpin ribozyme[9,10] consists of a 4H junction on which two adjacent arms have unpaired internal loops that interact to create the active site. The junction is antiparallel and left-handed in the crystal.[11] However, it is not known if this is the natural propensity of the junction alone or if it is forced by the interaction between the loops. We have therefore used the ribozyme as a basis for analyzing the handedness of the simple four-way junction derived from it, using electrophoretic experiments.

We study the electrophoretic mobility of double-stranded RNA species containing two axial bends that are symmetrically disposed about the center.[12,13] The overall shape of the molecule will be determined by the lengths of the three helical segments, the two bend angles, and by the dihedral angle (θ_D) relating the two outer helical segments. We can systematically vary θ_D by incrementally changing the spacing between the two bends. The electrophoretic mobility is a function of the overall end-to-end distance of the molecule, and this will be largely determined by the θ_D. An axial bend is created by a bulge comprising consecutive unopposed nucleotides.[12-16] As the spacing between the bends is varied, θ_D changes, and a sinusoidal modulation of electrophoretic mobility is normally observed. A duplex series with identical bends would have maximum and minimum electrophoretic mobilities for $\theta_D = 0°$ and $180°$ respectively, corresponding to spacer lengths of 11 or 22, and 5.5 or 16.5 bp, respectively (assuming standard helical parameters). When the directions of the two bends are not identical, the spacings corresponding to extreme mobilities will change, resulting in a phase change in the mobility modulation. If the direction of one bend is known, the change in phase can be used to calculate both the difference in direction of the bends and an absolute direction relative to the RNA helix.

Here we have exploited the structure of the hairpin ribozyme as a known bend, from which to characterize the A_4 bulge bend, and then used this bulge to measure the effect of ablating the loop-loop interactions between the arms to generate a simple 4H RNA junction (Figure 1). Ribozyme activity was substantially reduced by a G8U substitution.[17] The bending loci were symmetrically located about the center of the RNA, separated by a spacer of between 8 and 22 bp, with changes in the lengths of the outer arms to preserve the overall contour length at 88 bp, counting the ribozyme A-loop as 4 bp.

The $[\alpha\text{-}^{32}P]$-labeled species were electrophoresed in a 10% polyacrylamide gel in 90 mM Tris borate (pH 8.3), 5 mM Mg^{2+}
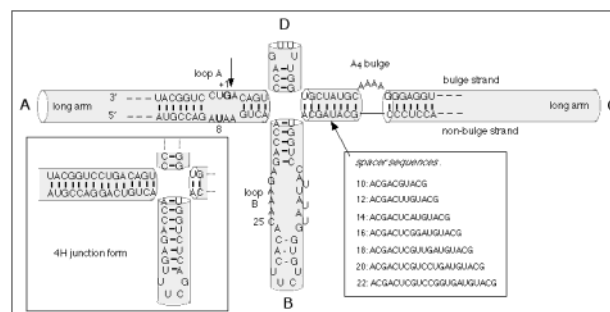

Figure 1. The double-bend RNA construct with the sequence of the hairpin ribozyme. (Left): A schematic of the constructs used to analyze bending trajectories. The two bending loci are the hairpin ribozyme junction and an A_4 bulge, shaded. These are connected by a spacer duplex of length that is varied from 8 to 22 bp in a series of species. The A and C arms are elongated, and thus the electrophoretic mobility is determined by the dihedral angle between them. (Right): The core sequence of the hairpin ribozyme and its junction. The position of cleavage in the active ribozyme is arrowed.
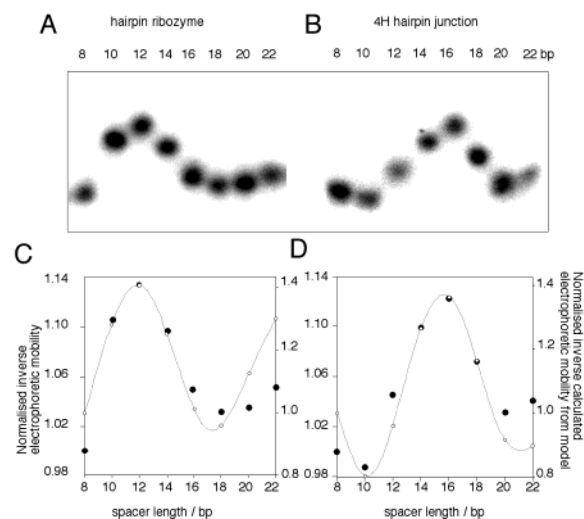

Figure 2. Electrophoresis of the hairpin ribozyme-A_4 bulge double-bend species in polyacrylamide gels. These experiments were carried out for constructs based upon the complete G8U hairpin ribozyme (A and C) and the simple 4H junction (B and D). The phosphorimages of the gels are shown in A and B; the tracks are labeled with the lengths of the spacer helices. Normalized inverse gel mobilities are plotted in C and D (filled circles) as a function of spacer length. The calculated normalized inverse gel migration values for the best fit model are also plotted (open circles and gray line).

ions at 20 °C (Figure 2A, B). Both sets of data exhibit a sinusoidal variation of electrophoretic mobility with bend-bend spacing, damped at longer separation. This is consistent with the obtuse angle subtended between the AD and BC axes in the average structures of both hairpin ribozyme-derived species in solution[18] and in the crystal.[11] However, visual comparison of the two patterns of mobility shows a significant change in the phase when the complete
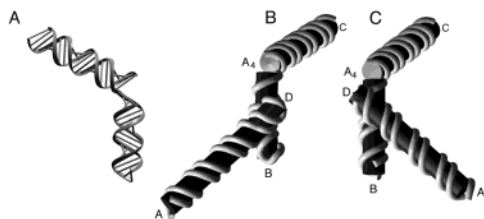
Figure 3. Cartoon representations of the geometry of the A_4 bulge, and the double-bend constructs. (A) The deduced bend direction of the A_4 bulge in an RNA helix. The direction is determined relative to the lower helix. (B) The double-bend construct based on the G8U hairpin ribozyme, with an 8 bp spacer helix. (C) The double-bend construct based on the 4H junction derived from the hairpin ribozyme, with an 8 bp spacer helix. Note the change in handedness between B and C.

ribozyme is replaced by its simple junction, with a shift of 4 bp in the spacer length that gives minimum mobility. This corresponds to a change in direction of 130° for a helical periodicity of 11 bp/turn, indicating that the relative direction of the axes is substantially changed when the loops are removed from the ribozyme.

Molecular graphics was used to construct models of these series of species to investigate the direction of bending. Helices were standard A-form geometry, and a bend angle of 65° was used for the A_4 bulge.[15,19] We initially introduced two bends with the same direction (ie zero phase shift) into an 88 bp duplex. From this we then constructed the series of species with different bend−bend spacing as used experimentally, with successive relative rotation of the outer arms. In the final stage a number of such series were generated, where a phase shift was introduced to represent changes in the direction of the junction bend in 10° increments. The end-to-end distance (d_{ee}) was measured for each of the modeled structures and related to electrophoretic mobility using the Lumpkin−Zimm[20] factors ($f_{LZ} = d_{ee}^2/L^2$), where L is the contour length. The values for each series were compared to the experimental mobilities to find the best fit of the maxima and minima (Figure 2C, D), thereby obtaining the direction of the A_4 bulge relative to the ribozyme/junction and the central RNA helix (Figure 3A).

The A_4 bulge was used to deduce the structure of the isolated 4H junction derived from the ribozyme, based on the observed 130° phase shift on removal of the loops. A direct and simple approach to modeling the change in the junction was used. By rotating θ_D until a phase change of 130° was obtained, we obtained a direction for the terminal arm of the junction. However, there was no local structural information that would explain how the center of the four-way junction would change to achieve this. A simple reversal of handedness alone would naively predict a phase shift of 180° rather than the 130° observed experimentally. However, close inspection of the hairpin ribozyme junction shows that the loop−loop interactions generate significant axial kinks in both the A and B arms. When removed by complementation, these will revert to simple A-form helices and thus straighten. We therefore altered the existing double-bend model by retaining the A_4 bend geometry, straightening the axes of the former loop regions, and then applying a pivot to invert the chirality. This then gave a good fit to the experimental data (Figure 2D), with a corresponding 130° change in phase.

Thus, the simplest interpretation of the electrophoretic data involves a change in handedness between the complete ribozyme (Figure 3B) and the simple four-way junction (Figure 3C). This indicates that the strong interaction between the loops of the

ribozyme is responsible for inverting the natural chirality of the four-way junction. Our conclusion that the ribozyme possesses a chirality opposite to that of the simple junction was tested by the construction of a new series of RNA molecules identical to the previous ribozyme/A_4 bulge constructs except that the loop−loop interactions were disrupted not by complementation but by a single mutation. In the intact ribozyme G+1 is extruded from loop A and inserted into a pocket within loop B, where it basepairs with C25.[11] Folding of a G+1A ribozyme is severely impaired.[17,21] A sinusoidal modulation of electrophoretic mobility was observed with a phase essentially identical to that observed using the species from which the loops had been completely ablated (Figure S1). We conclude that presence or absence of the loops has no effect on the conformation of the junction unless they interact.

The straightforward interpretation of our experiments is that there is a change of junction chirality when released from the interaction between loops, from the left-handed conformation observed in the crystal structure of the complete ribozyme[11] to a right-handed conformation in the simple junction. This indicates that the free 4H RNA junction has the same handedness as its DNA equivalent.[4−6] Single-molecule fluorescence experiments have shown that the junction acts as a folding-enhancer in the natural hairpin ribozyme.[22] Our results suggest that the folding trajectory of the hairpin ribozyme probably involves a change in chirality of the junction in the course of the formation of the active loop−loop interaction.

Supporting Information Available: Plot of data for G+1A ribozyme, full sequences, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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