A small modified hammerhead ribozyme and its conformational characteristics determined by mutagenesis and lattice calculation

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ABSTRACT

A prototypic hammerhead ribozyme has three helices that surround an asymmetrical central core loop. We have mutagenized a hammerhead type ribozyme. In agreement with previous studies, progressive removal of stem-loop II from a three stemmed ribozyme showed that this region is not absolutely critical for catalysis. However, complete elimination of stem II and its loop did reduce, but did not eliminate, function. In a stem-loop II-deleted ribozyme, activity was best preserved when a purine, preferably a G, was present at position 10.1. This G contributed to catalysis regardless of its role as either one part of a canonical pair with a C residue at 11.1 or a lone nucleotide with C (11.1) deleted. Computational methods using lattices generated 87 million three-dimensional chain forms for a stem-loop II-deleted RNA complex that preserved one potential G-C base pair at positions 10.1 and 11.1. This exhaustive set of chain forms included one major class of structures with G(10.1) being spatially proximal to the GUCX cleavage site of the substrate strand. Strong correlations were observed between colinear arrangement of stems I and III, constraints of base-pairing in the central core loop, and one particular placement of G(10.1) relative to the cleavage site. Our calculations of a stem-loop II-deleted ribozyme indicate that without needing to invoke any other constraints, the inherent asymmetry in the lengths of the two loop strands (3 nt in one and 7 nt in the other) that compose the core and flank G10.1–C11.1 stipulated strongly this particular G placement. This suggests that the hammerhead ribozyme maintains an asymmetry in its internal loop for a necessary structure/function reason.

INTRODUCTION

Hammerhead ribozymes represent one class of catalytic RNAs (1–5). Secondary structures that include three RNA-helices surrounding a central core region were originally suggested by

phylogeny and later confirmed by extensive mutagenesis (6–10). Recently, an X-ray crystal structure has been determined for a 47 nucleotide (nt) hybrid RNA–DNA ribozyme analog, in which the substrate strand is a 13 nt DNA fragment (11). A model three-dimensional structure has also been determined by fluorescence resonance energy transfer (FRET) for a fluorophore substituted 43 nt two-stranded hammerhead (12). In both approaches, the ribozyme structures show a narrow Y-shaped form, wherein the helical axes of stems I and II make similar obtuse angles relative to the helical axis of stem III. Inspection of the two structures indicates that either of the two pairs of stems that include stem III may be considered to be approximately colinear.

There are some computational approaches to model three-dimensional RNA structures (13). One straightforward approach utilizes sets of connected double helical stems assembled to be consistent with distances and other experimentally determined constraints (14–16). Development of models from FRET studies of hammerhead RNAs is one example of such a constraint-based method (12). Once initial models are generated, energy minimization methods are applied to refine further the structures (17,18). A second approach is to search configurational spaces for selected geometries found in a small library of RNA crystal structures (19).

Lattice models were developed recently to model structural motifs from various proteins and t-RNAs (20–22). In this approach, as applied to t-RNA, each lattice point is chosen to represent an O3' atom. Rigid volumes representing stems are attached to the lattice points that correspond to the O3' atoms of the nucleotides of the t-RNA internal loop. All possible internal loop forms and their attendant stem orientations can then be computationally generated, accounting for excluded volumes. The resulting forms are evaluated to determine patterns of stem–stem arrangements and other structural features. Because the complete enumeration of all folds is possible using lattice methods, the approach avoids the uncertainties of multiple energy minima and other issues inherent to less extensive methods. Furthermore, the exhaustive sampling provided by these methods significantly reduces the impact of possible biases that might be present in a more limited search. The validity of lattice methods was demonstrated for

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t-RNA, with successful prediction of native tertiary pairs and stem arrangements (22).

Here, we have modeled a small ribozyme using lattice methodology. Because we are interested in the minimal elements that preserve catalysis, and because it is difficult to verify reduced activities reliably, we used an iterative method (23) to assess low levels of cleavage of substrate RNAs. Our assays were not designed to quantitate kinetic cleavage data, instead we wished to verify whether a hammerhead ribozyme that was eliminated for stem II still maintained measurable activity. Hence, the iterative approach increases measurement sensitivity in that it sums cleaved products that are accumulated over many cycles of substrate-enzyme interaction. Thus, we could demonstrate activity in a hammerhead ribozyme which was deleted for all traces of stem-loop II. We were also able to determine that a purine, most notably G, at position 10.1 contributed significantly to activity. Furthermore, our lattice calculations of this small model ribozyme revealed insights into (i) the flexibility of the hammerhead core that permits certain essential bases to approach the substrate cleavage site; (ii) the correlation between colinearity of stems I and III and potential base-pairings within the core loop and (iii) the contribution of core loop asymmetry in determining a functional ribozyme structure.

MATERIALS AND METHODS

Plasmids and oligonucleotides

The template plasmid for *in vitro* transcription of the RNA substrate contains an HIV-1 LTR-CAT positioned into the pGEM4Z vector downstream of the T7 promoter (pT7LTR-CAT). The RNA strands for ribozyme were transcribed from synthetic oligonucleotides pairs with T7 promoter sequence attached at the 5' terminus.

In vitro transcription/ribozyme cleavage

pT7 LTR-CAT was linearized with *EcoRI*, 495 bases from the transcription start site. Synthetic oligonucleotide derived ribozyme RNA strands were gel purified and then annealed in buffer (200 mM Tris pH 8.0, 250 mM NaCl) by heating in a 65°C water bath for 3 min and then cooling sequentially at 55, 42 and 37°C for 2 min each. $MgCl_2$ was added to 20 mM to initiate reaction. To monitor cleavage, substrate RNA was radiolabeled with [α - ^{32}P]CTP. Cleavage reactions were conducted pair-wise, in parallel, with one-half of the reactions subjected to repeated thermal cycling (23). These reactions were carried through 10 cycles of heating to 80°C for 1 min followed by 37°C for 6 min. Mineral oil overlay was used to minimize evaporation during the heating cycles. The other half of the reaction mixtures was annealed step-wise as described above and then kept at a constant 37°C. Reaction mixtures were terminated with an excess of EDTA-containing formamide-stop solution added at the end of the reaction time period. Samples were maintained on ice until loaded for electrophoresis.

Electrophoretic analysis of ribozyme reactions

After 10 thermal cycles (~90 min) or an equivalent amount of parallel incubation time at a constant 37°C, both sets of reactions were halted. All samples were denatured by heating at 95°C for 3 min followed by rapid chilling on ice. Reaction aliquots, along with radiolabeled pBR322-*HpaII* molecular size markers, were resolved in an 8% denaturing polyacrylamide gel containing 8 N

urea. The cleavage products and uncleaved substrates were visualized by autoradiography, and were quantitated using a Fujix Bas2000 Bioimaging analyzer.

Lattice modeling

Each lattice point of the internal loop represented an O3' atom. Attached to the internal loop were two rigid volumes that represented the stems of the minimal hammerhead. The volume exclusion is made implicit in forbidding multiple occupancy of the loop lattice points and in the model stems. The model stems are taken as elements of solid volumes fitted to an idealized all atom A-form helical stem of 7 bp. This was the longest stem for which it was feasible to completely enumerate all lattice forms of the internal loop with all the relative orientations of attached rigid stems. The overall pattern of folding is not affected by using these model stems.

The cubic lattice was used as the simplest representation that permitted the complete enumeration of forms. The model of the double helical A-form stem has a square cross-section because a cubic lattice is utilized. Although this device does not provide a completely realistic representation of the curved surface of the helix, it does divide the helix surface into flat plates that are useful for approximate assessments of the location of external elements. The cubic lattice method used here has the helical axes parallel or perpendicular with respect to one another. Hence, pairs of stems involved in the Y-shaped ribozyme form are approximated as being colinear. As with any lattice method, the resulting structures are approximations; although overall folding patterns are preserved.

RESULTS

A hammerhead ribozyme deleted in stem-loop II

A prototypic hammerhead ribozyme has three helical stems, an internal loop and a hairpin loop at stem II. In constructing a lattice model for hammerhead ribozyme, we wished to account for elements essential for chemical catalysis. Sequence requirements for a hammerhead ribozyme have been characterized extensively (10,24, and references cited in 2). From previous findings, it is clear that helix-stem I and helix-stem III (see Fig. 1A) serve an obvious function in hybridizing a substrate RNA strand to a second enzyme RNA strand. Similarly, sequences in the catalytic core of the enzyme strand serve necessary roles in chemical catalysis and in binding to Mg^{2+} (25). However, at the time that we initiated this work, stem-loop II was originally regarded to be important but without a specifically assigned function, but two recent studies have addressed roles for stem-loop II (26,27).

Our attempts to dissect the contribution of stem-loop II to chemical catalysis were complicated in two ways. First, we found that certain changes to stem-loop II drastically reduced ribozyme activity, often to a degree that made reliable detection of cleavage suspect. Secondly, we were concerned that differential effects from various perturbations of stem-loop II sequence would confuse readouts in assays that failed to normalize for rate-limiting constraints (from stem I and stem III) at the product release step (26). To resolve these two issues, we used an iterative assay based on multiple rounds of thermal cycling (23) that measures cleaved substrate RNA accumulated over a specified time. Conclusive determinations of low-level catalysis are critical for developing valid computational models, which are restricted to smaller sequences. Because poorly-detected cleavages from each

Figure 1. Comparative assay of six different ribozymes that have different length in stem-helix II. Substrate strand was at 10 nM, and the two RNA strands that compose the enzyme loop were at 1 nM. (A) Schematic representation of the sequences and secondary structures of mutagenized forms of hammerhead ribozyme (top), with quantitation of cleavages after 90 min of incubation from the average of four experiments tabulated below (bottom). In the schematics of the ribozymes, only the 28 nt portion of the substrate RNA that is complementary to the enzyme RNA strand is shown. (B) Autoradiogram of one representative cleavage assay without (left) and with (right) thermal cycling. Arrow points to the 113 nt (smaller) cleavage product generated from the starting radiolabeled substrate (largest band in each lane). Left panel was exposed for 10 times longer than the right panel. The bottom insets are overexposures of the respective gel regions that contained the 113 nt cleavage product.

cycle are summed over multiple cycles, small catalytic activities are amplified and thus quantitated with reliability (Fig. 1B). Furthermore, product-release was facilitated at the end of each cycle by a thermal step elevation to 80°C; which normalizes for differences in rate-limiting effects due to sequence size for various mutants.

Results in Figure 1 serve to illustrate some of the above points. We constructed six variants of a prototypic hammerhead ribozyme (Fig. 1A). Each contained the same 28 nt RNA sequence in a larger substrate strand but differed from one-another in that the two RNA strands that comprise the internal core loop, after annealing, formed stem IIs of different lengths (no mutant had a stem II loop; Fig. 1A). Figure 1B shows one typical comparative cleavage assay of mutants (with average quantitation from four experiments shown at bottom of Figure 1A), without (left) or with (right) thermal cycling. Both sets of reactions were incubated for the same total duration (90 min). Two findings were instructive. First, repeated temperature cycling did increase detection sensitivity by ~10-fold (left panel was exposed to film 10 times longer than right panel). Hence, cleavage products from low-activity RNAs (e.g. the '1 bp' ribozyme) were more clearly characterized with (Fig. 1B, lane 10) than without (Fig. 1B, lane 3) thermal cycling. Secondly, we observed a distinct difference in the relative behavior of the '20 bp' ribozyme (when compared with

counterpart '4 bp' or '10 bp' ribozymes) in the two assay formats (Fig. 1B, lanes 7 and 14). For instance, in comparing the 113 nt cleavage product (arrow, Fig. 1B) with the '10 bp' versus the '20 bp' ribozyme, we found that the ratio was >10 without thermal cycling (Fig. 1B, left panel; ratio of lane 6 to lane 7) and was essentially 1 with thermal cycling (Fig. 1B, right panel; ratio of lane 13 to lane 14). The counterpart reactions in panels A and B, except for thermal cycling, were otherwise conducted identically. Hence, one interpretation is that thermal cycling does normalize for differences in the product-release step that might otherwise exist between the '10 bp' and the '20 bp' RNAs. We noted additionally that the continuation of significant activity with progressive deletions up to the last nucleotides at positions 10.1 and 11.1 suggested an intact terminal G-C pairing.

The above results validated the use of thermal cycling in characterizing low activity ribozymes. Hence, we first used this approach to study the consequences on catalysis of changes engineered into the internal loop (highlighted by shading in Figure 2A) of a stem-loop II-deleted ribozyme. Initial results on altering sequences within the loop were disappointing with virtually all changes abolishing activity completely (N. H. Lin, unpublished observations). However, systematic examination of each residue in the internal loop revealed that the base positions 10.1 and 11.1

Figure 2. Relative cleavage activities from a minimal model ribozyme that contain different nucleotide substitutions at positions 10.1 and 11.1. (A) Schematic representations with relative activities indicated below. The activity of the G-C pair was arbitrarily set at 1. (B) Autoradiogram showing a typical cleavage assay. For comparisons, normalized percent of cleavage was obtained based on the amount of 113 nt product (arrow) as quantitated by a phosphorimager. Comparisons represented the averages from four separate assays.

tolerated certain changes (see example assay in Fig. 2B). Based on averages from four separate assays, we were able to establish a hierarchical order of relative cleavages for 17 variant ribozymes (see Figs 2 and 3).

Catalysis observed for many of these three-stranded variants (Figs 2 and 3) deleted for stem-loop II suggests that these molecules retain some of the secondary and tertiary hammerhead features needed for activity. Indeed, other studies using NMR and CD measurements of a 39 nt, catalytically active, three-stranded ribozyme revealed structural features in that RNA complex (28) consistent with those found in a prototypic two-stranded hammerhead, and agree with our results that the loop sequence of stem II contributes little to activity. Figures 2 and 3 show a diminishing hierarchy of activity for the canonical pairings of G-C, G-U and A-U at positions 10.1 and 11.1. Possible non-canonical pairings of A-C, G-A and G-G in Figures 2A and 3A had even lower activity. It seems clear that if there is a G at position 10.1, then the canonical pairs are preferred (Fig. 3A). Interestingly, we saw no evidence that simultaneous presence of bases at both 10.1 and 11.1 was needed for catalysis; a lone G(10.1) showed activity (Fig. 3C) quite comparable with G-C at positions 10.1 and 11.1). Whereas a positional reversal of pairing (i.e. C at 10.1 and G at 11.1; Fig. 3B) or an absence of a purine at position 10.1 (Fig. 3D) showed no catalysis, indicating a position specificity for G.

Substituting an A at position 10.1 maintained a lower function (Fig. 3C).

Lattice modeling of ribozyme

Because a three stranded minimal ribozyme with a single G-C pair in stem-loop II was active, lattice calculations were performed for an abbreviated version of this complex (with bases shown in color; Fig. 4A). We enumerated all the possible lattice forms of the 11 nt internal loop for the packing of the two stems and the single G-C pair at positions 10.1 and 11.1. Three-dimensional representations of a colinear stem structure are shown in Figure 4B-D. Each nucleotide of the internal loop is represented (as shown in the lattice representations in Fig. 4C and D) by a point on a cubic lattice and connected to other lattice points by virtual bonds of 6.15 Å in length. The A-form helical stems are represented as solid volumes, with cubic lattice spacing of 6.15 Å, and were taken from the lattice fit to an ideal 7 bp A-form helical stem (see stem I and stem III; Fig. 4C and D). In this fit, the relative positions of attachment sites for the stems, including the tilted A-form orientation of stem bases, are preserved.

The calculations resulted in 86, 764, 520 chain forms. Fig. 5 shows the distance distribution in these forms between G(10.1) of one internal loop RNA strand (see red chain in Fig. 4) and the

Figure 3. Additional comparative cleavages from ribozymes with the indicated changes (A–D) at positions 10.1 and 11.1. Open dotted circle indicates a missing nucleotide. All assays were normalized against the activity of the model ribozyme that contain a G-C pair at positions 10.1 and 11.1 arbitrarily set to 1.

cleavage site at C(17) in the substrate strand (see purple chain in Fig. 4). About 30% of the folds have G(10.1) proximate to C(17), when 24 Å was used as the upper bound for defining proximity (22). In Figure 4C and D are representations of a form consistent with the most common loop orientation relative to the cleavage site, with two views rotated 180° around the helical axis. The G(10.1) and other loop nucleotides are found closest to the prism surface 3 that is inclusive of both loop attachment sites. This form is also an example of a colinear stem arrangement in which ~7% of all the forms have such colinear stem arrangements.

Loop constraints

The single G-C pair at positions 10.1 and 11.1 is unlikely to be stable as an isolated base pair. Three other adjacent base pairs, U7-A14, G8-A13 and A9-G12, were observed in the internal loop

of related hammerheads (11,28). Corresponding constraints were imposed in one set of our calculations by requiring the distances between the lattice points representing the three pairs to be within the range from 10.65–20.40 Å, consistent with nucleotide pair distances of the cubic lattice structure fit to the Phe t-RNA (22). We first considered the fraction of forms, either with or without these constraints, that have a particular stem face (see Fig. 4C and D) closest to G(10.1). Only three faces of stem I are found truly accessible to this G residue. These include face 1 (the surface at the end of the stem adjacent to the internal loop); face 3 (the surface inclusive of the two internal loop attachment sites) and face 5 (the other stem surface adjacent to the 3' attachment site). For the 22% of forms that conformed to the U7-A14, G8-A13 and A9-G12 distance constraints, an increased fraction (11%) had colinear stems as compared to a smaller fraction (7%) having colinear stems out of all possible forms.

Table NO TAG summarizes the fraction (with or without the base-pairing constraints) of folds with G(10.1) being proximate to the cleavage site on the substrate RNA. The effect of imposing colinear stems and loop base-pairings reduced enormously (from 25 764 691 to 276 810) the number of loop forms that contained a proximate G(10.1). This reduction by a factor of 93 illustrated how significantly these parameters restricted loop foldings. Interestingly, it also indicated that despite these restrictions there remained a remarkable lack of rigidity permitting alternative forms that are presumably associated with catalysis. Hence, this suggests a flexibility to the structure of the hammerhead ribozyme for catalysis that might not be observed in more rigid ribozymes such as hairpin (29,30).

An accompanying result of specifying colinearity of the two stems is to significantly increase loop access to stem face 3, and to reduce access to stem face 1. This is because colinear stem arrangements squeeze out loop nucleotides from the space between the two stems. Requiring the U7-A14, G8-A13 and A9-G12 base-pairing constraints did lead to additional access by G(10.1) to stem face 3. Other constraints, such as the C3-C17 pairing and G5 being proximal to the 5' internal loop attachment site which were described in the resolved X-ray crystal structure for a catalytically active 47 nt RNA–DNA hammerhead analog (11), did not change significantly the pattern of the results in Table NO TAG (Lustig, unpublished observation). Additional analysis showed that the dominant loop orientations for G(10.1) and for the average loop position, with and without any constraint on G(10.1) position relative to the cleavage site, are the ones closest to stem face 3 (the surface that included both loop attachment sites).

Table 1.^{a,b} Fraction of forms for selected stem face closest to G(10.1)

	Faces ^c of Stem 1			
	Face 1	Face 2	Face 3	Face 4
With colinear stems and constraints (276 810 forms)	0.18	0	0.82	0
No constraints (577 548 forms)	0.21	0	0.79	0
With other stem arrangements and constraints (3 597 536 forms)	0.43	0	0.52	0.04
No constraints (21 312 797 forms)	0.35	0	0.62	0.04

^aForms where G(10.1) is <24.0 Å from cleavage site.

^bThere are 25 764 691 total forms included here.

Figure 4. Three-dimensional representations of model three-stranded ribozyme deleted in stem-loop II. (A) Secondary structure representation of a minimal hammerhead ribozyme. Model 7 bp stems are in color; 'purple' is the substrate strand, and the two loop-composing strands are colored 'blue' and 'red'. A single G-C pair is indicated at positions 10.1 and 11.1. (B) Schematic illustration, a three-dimensional ribbon representation, of a minimal ribozyme consistent with a common form that includes colinear stems. 'Purple' is substrate strand, and 'red' and 'blue' are the two pieces of RNA that compose the internal loop. (C) Lattice structure showing colinear stems (stems shown as solid blocks). (D) Perspective of Figure 4C that is rotated 180° around the helical axis. The prism faces of stem I are circled as 1-6 in C and D.

Figure 5. Distribution of the distances between G(10.1) and the cleavage site for the 86 764 520 lattice forms. Shaded region indicates those forms with distance <24.0 Å.

^cNo forms have proximal G(10.1) closest to stem faces 5 and 6.

DISCUSSION

A minimal three-stranded ribozyme

There are suggestions that stem-loop II stabilizes the transition state and assists in forming the correct tertiary structure through base-pairing (6,26). Because neither the sequence nor the length of stem-loop II needs be strictly conserved in order to maintain catalysis (7,10,26,27,31,32), it is suggestive that this portion of the ribozyme contributes more towards structure than to direct participation in cleavage. The exhaustive enumeration of forms by lattice affords perspectives that might not be revealed by other approaches. Since this type of extensive computation is feasible only for small RNAs, we first attempted to define experimentally small RNA complexes that retained activity. Hence mutagenesis was designed with the aim of maximizing reduction of sequences, understanding that experimentally derived minimal forms might not retain all characteristics of the original hammerhead ribozymes.

Our experiments did reveal that reproducible, albeit reduced, catalysis was observed for structurally open three stranded ribozymes that were deleted for stem-loop II. In agreement with others (10,26,29), we found that bases at the terminal positions of stem II were required for appreciable activity with G(10.1)-C(11.1) pairing contributing to activity even in a setting where the rest of stem-loop II was missing. This finding perhaps reflected more of a key role for a purine at position 10.1 than for pairing *per se*, since a C(10.1)-G(11.1) base-pair in our assay (Fig. 3B) and in assays by others (26) was found to be highly ineffective for catalysis.

Role of G(10.1)

In our stem-loop II-deleted ribozyme, a purine residue at position 10.1 was important for catalysis. (G was more effective than A, whether base-paired or present alone.) Mechanistically, the possible role of purine and its N7 moiety forming a Mg²⁺ binding site has been proposed for a two-stranded RNA-DNA hammerhead (11), and this would be compatible with our results. Indeed,

the similarities in cleavage seen for a complex competent for G-C pairing at positions 10.1 and 11.1 and one with a lone G(10.1) suggest that other nearby pairings [i.e. U7-A14, G8-A13, A9-G12, as observed in the RNA-DNA ribozyme (11)], have greater importance in stabilizing local structure. The paradigm of enzyme mechanisms leads to the expectation that critical functional groups, that are not too rigid to preclude motion, be placed inside a somewhat rigid structural pocket. By analogy, one might envision that all or a part of G(10.1) and/or additional loop nucleotides be flexible, albeit within a highly structured larger environment (11).

Core ribozyme structure

The calculations support the intuition that colinear stem arrangements usually squeeze out the internal loop from the space between the two stems. This permits easier access of G(10.1), in the preferred loop orientation, to the substrate cleavage site. In addition, the constraints of the three additional pairs, U7-A14, G8-A13 and A9-G12, in the internal loop increase by >50% the fraction of possible forms with colinear stems, which also can enhance the possibility of the preferred loop orientation. However, requiring forms that have colinear stems also to have these base-pairings only modestly increases the exclusion of portions of the loop from the region between the stems. The combination of colinear stems and base-pairing constraints does result in one particularly dominant placement of residue 10.1 relative to the cleavage site. Indeed, the correlation that we noted here between stem arrangement and the correct placement of the internal loop has also been suggested by Tuschl *et al.* (12).

However, it is noteworthy that the same preferred placement is found in 35% of the cases even without consideration of stem arrangement or loop base-pairings. This suggests the importance to catalytic function of the asymmetry in length (7 nt on one side and 3 nt on the other) of the internal loop RNAs flanking the base-pair at positions 10.1 and 11.1. This may also apply similarly to wild-type hammerheads, as well; and provides a possible functional explanation for the evolutionary preservation of an asymmetrical configuration for the internal loop. This preferred orientation of the internal loop describes a possible avenue of access for the loop to interact with the cleavage site during catalysis.

Lattice approach

Our extensive calculation of over eighty million forms is a relatively unbiased search of the configurational space of possible chain forms. The absence of any experimental three-dimensional structures for a three-stranded minimal (i.e. stem-loop II-deleted) ribozyme makes this sort of calculation useful. The advantage of this exhaustive lattice approach over other computational methods is that it is not limited *a priori* by the intrinsic limitations associated with energy derived computational outcomes (33) and incomplete criteria for RNA. Such problems include flawed restrictions of all-atom conformational space determined from the limited set of RNA crystal structures. In addition, other modeling methods are not exhaustive in their searches so a suggested low energy structure may mistakenly involve a local energy minima (34). Because lattices describe simplified representations of all atoms in a regular pattern, efficient algorithms can be written that allow complete searching of chain space. This simplified coarse grained representation of molecular subunits and their bonds has been useful in

modeling thermodynamic and related physical properties of polymers, including biomolecules (20,21,35–39).

Similar insights have been characterized by two types of lattice methods for Phe t-RNA (22), and these insights revealed that the most common predicted stacking arrangements for t-RNA was the native two pairs of stacked stems found in the Phe t-RNA crystal structure (40). The calculations also successfully identified the regions of largest flexibility for Phe t-RNA and predicted alternative three-dimensional forms, including the one that was subsequently defined (41) by experiments. In those lattice calculations, one also found that the correct stacking of one pair of stems had a tendency to mandate the configuration of the other pair.

We believe that the application of the lattice method to our minimal ribozyme was useful because it helped explain the current experimental results in the absence of any specific X-ray crystal or other three-dimensional data for the model stem II-deleted hammerhead. The calculations showed that constraining G(10.1) by canonically pairing it to C(11.1) as well as by the presence of adjacent pairs affords significant access for G(10.1) to the cleavage site. As was suggested by the calculations, additional stabilization from adjacent pairs, including G8-A13 and A9-G12, also appears to enhance the probability of a particular stem arrangement and resulting loop orientation involving G(10.1). These constraints are consistent with X-ray, FRET and other experimental results for the two-stranded hammerhead (11,12,28). Evidence of these constraints in the minimal three-stranded hammerhead suggest structural similarities with the two-stranded hammerhead.

Conclusions

Consistent with the results of Tuschl and Eckstein (26), under conditions that favor the cleavage step, a minimal three-stranded hammerhead ribozyme does not absolutely need stem-loop II for catalysis. The presence of a purine position at 10.1, preferably a G, either paired canonically or unpaired is important for significant activity. The catalytic activity of a ribozyme with an unpaired purine at 10.1 suggests that adjacent base pairs in the internal loop (which were observed in the crystal structure of a RNA-DNA two-stranded hammerhead) provide stabilization for the reaction precursor. Our calculations suggest that the three-stranded minimal hammerhead, even with conditions of colinear stems and the three base-pairings in the internal loop, lack significant rigidity, and that there is a possible preferred orientation of the loop relative to stem I. This orientation becomes more likely when stems I and III are colinear and when U7-A14, G8-A13 and A9-G12 are base-paired in the internal loop.

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