Modifications and deletions of helices within the hairpin ribozyme–substrate complex: An active ribozyme lacking helix 1

ROBERT PINARD,¹ DOMINIC LAMBERT,^{1,2} GULNAR POTHIAWALA,¹ FRANÇOIS MAJOR,² and JOHN M. BURKE¹

¹Markey Center for Molecular Genetics, Department of Microbiology and Molecular Genetics, The University of Vermont, Burlington, Vermont 05405, USA

²Département d'Informatique et Recherche Opérationnelle, Université de Montréal, 2920 Chemin de la tour, C.P.6128, Succ. Centre-ville, Montréal, Québec H3C3J7, Canada

ABSTRACT

Within the hairpin ribozyme, structural elements required for formation of the active tertiary structure are localized in two independently folding domains, each consisting of an internal loop flanked by helical elements. Here, we present results of a systematic examination of the relationship between the structure of the helical elements and the ability of the RNA to form the catalytically active tertiary structure. Deletions and mutational analyses indicate that helix 1 (H1) in domain A can be entirely eliminated, while segments of helices 2, 3, and 4 can also be deleted. From these results, we derive a new active minimal ribozyme that contains three helical elements, an internal loop, and a terminal loop. A three-dimensional model of this truncated ribozyme was generated using MC-SYM, and confirms that the catalytic core of the minimized construct can adopt a tertiary structure that is very similar to that of the nontruncated version. A new strategy is described to study the functional importance of various residues and chemical groups and to identify specific interdomain interactions. This approach uses two physically separated and truncated domains derived from the minimal motif.

Keywords: truncated domains; RNA; molecular modeling; catalytic structure; structural elements

INTRODUCTION

The hairpin ribozyme catalyzes a reversible RNA cleavage reaction and has a simple secondary structure, consisting of two domains, each containing an internal loop and two short helices. The active site is comprised of multiple conserved functional groups that are located at and around the interface of the two domains in the docked, active tertiary complex (Hampel et al. 1998; Pinard et al. 2001a; Rupert and Ferré-D'Amaré 2001).

The tertiary structure of the ribozyme–substrate complex has been examined using a variety of biochemical, biophysical, genetic, and computational methods (Walter and Burke 1998; Lilley 1999; Fedor 2000; Walter 2001). Tertiary structure formation is accompanied by a large conformational

change within both internal loops. The conformational change can be visualized by comparing structures of the isolated domains, obtained by NMR spectroscopy (Cai and Tinoco 1996; Butcher et al. 1999), with that of the complete complex, obtained through X-ray crystallography (Rupert and Ferré-D'Amaré 2001). It can also be monitored using time-dependent assays, including fluorescence resonance energy transfer, hydroxyl radical footprinting, and photocrosslinking (Walter et al. 1998; 1999; Pinard et al. 1999a; Hampel and Burke 2001).

Two key interactions stabilize the tertiary structure. The G + 1 · C25 base pair was identified by hydroxyl-radical protection, covalent crosslinking, molecular modeling, and compensatory base substitutions (Pinard et al. 1999b). A conventional ribose zipper links the N3 atoms of A10 and A24 with the 2'-hydroxyl groups of nucleotides 10, 11, 24, and 25 (Earnshaw et al. 1997). Each of these interactions has been confirmed by the crystallographic structure (Rupert and Ferré-D'Amaré 2001), and is observed in detailed models of the ribozyme–substrate complex that we have recently developed (Pinard et al. 1999b, 2001a,b).

Reprint requests to: John M. Burke, Markey Center for Molecular Genetics, Department of Microbiology and Molecular Genetics, The University of Vermont, Burlington, VT 05405, USA; e-mail: john.burke@uvm.edu.

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Together, these results have provided a wealth of information on the spatial organization of the ribozyme–substrate complex. Nevertheless, our ability to dissect the conformational change and to probe the functional importance of these interactions is limited. In the present study, we present an analysis of the effects of systematic deletions extending through helices 1 and 2 in domain A, and mismatches in helix 3 of domain B. Our results lead to the derivation of a new active minimal ribozyme, in which helix 1 is absent.

RESULTS

Deletions and mismatches in helices 1–3: Helix 1 is nonessential

To ascertain the importance of base pairs in helices 1 and 2, we measured the catalytic activity of several constructs containing deletions throughout this region (Fig. 1). Two series of deletions in the substrate were generated, in which nucleotides were removed one at a time from the 3' and 5' ends. To evaluate the binding affinity of the hairpin ribozyme for these truncated substrates (H1 Δ 1–6 and H2 Δ 1–

4), apparent K_D s were determined using an electrophoretic mobility-shift assay (Fig. 2 and Materials and Methods). Rate constants for the cleavage reactions were determined under single-turnover conditions, with three ribozyme concentrations, each well above the apparent K_D .

Results showed that the deletions in H1 progressively reduced the stability of the ribozyme–substrate complex, as expected (Fig. 2A). Deletion of successive nucleotides from the 3' end of the substrate (H1 Δ 1, Δ 2, Δ 3) resulted in modest increases of cleavage rates in the single-turnover reaction, while further deletion of nt 5 and 6 (H1 Δ 5, Δ 6), reduces cleavage rates by a factor of approximately 100. These results lead to the conclusion that helix 1 can enhance cleavage activity, but is not an essential component of the hairpin ribozyme.

Progressive deletions within helix 2 showed that the two substrate nucleotides distal to the cleavage site make little or no contribution to cleavage activity at saturating substrate concentrations, while the two nucleotides proximal to the cleavage sites are of much greater importance (Fig. 2). However, analysis of the dissociation constants demonstrates that base pairs at the two positions of H2 distal to the cleavage site contribute moderately to the stability of the ribozyme–substrate complex.

In addition to the standard ribozyme (Fig. 1), we also employed a circularly permuted construct, in which the 5' end of the substrate binding strand (ribozyme position 1) was linked to the 3' end of the ribozyme (position 50). This construct was designed to constrain domain A to a conformation similar to the conformation proposed by molecular modeling studies (Pinard et al. 2001b). Using the circularly permuted ribozyme, the cleavage activity of substrates containing 3' deletions showed a pattern that was similar to that obtained using the standard ribozyme, although cleavage rates for active species were uniformly lower (data not shown). A similar trend held for the 5' deletion series, except that the Δ 3 construct was more severely inhibited using the circularly permuted ribozyme (data not shown).

We introduced a series of mismatches into the end of helix 3 located at the helical junction (Fig. 3B). Activity is slightly increased by a single mismatch, whereas two or three mismatches give rise to strong inhibition. No cleavage activity could be detected in a construct having four mismatches. Binding assays showed that the loss of activity by these mismatches was associated with a reduction in the ability to form a stable domain B (Fig. 3B).

FIGURE 1. Ribozyme–substrate complex. Secondary structure of the complex between the hairpin ribozyme and its cognate substrate used in this study. Arrow indicates substrate cleavage site. Substrate nucleotides are numbered from the cleavage site with positive and negative signs. Ribozyme nucleotides are numbered sequentially from the 5' end. H1–H4 denote the four helical elements of the complex. Sequences where deletions were introduced are marked with gray shadows, and sites where mismatches were introduced are marked by gray circles. Nucleotides participating in the critical G + 1 · C25 interdomain base pair are indicated by solid black circles, and G8/A-1 stacking is marked by a line. Sequences whose presence is required in minimal ribozyme construct are enclosed by gray rectangles.

Combinations of mismatches and deletions: Register of domain alignment

The effects of combination of mismatches and deletions were explored, and results are shown in Figure 3A. In helix 1, deletions of the ribozyme strand, the substrate strand, or both strands were observed to either slightly enhance or not to affect cleavage activity. In particular, the constructs

FIGURE 2. Cleavage activity of truncated variants in helices 1–3. Cleavage assays were performed with constructs having a linear or a cyclized substrate binding strand (SBS) and bearing a sequence of deletions and/or mismatches in helices 1, 2, and 3. Substrate-binding strand denotes sequences corresponding to nucleotides 1–14 of the ribozyme (Fig. 1). Results for constructs with a linear SBS are shown in black; those for constructs with a cyclized SBS (in which nt 1 and 50 are directly linked) are shown in white. Cleavage rate measurements were carried out at saturating conditions under single turnover conditions. Values for apparent K_D were determined by gel mobility-shift assays as described in Materials and Methods. K_D measurements monitor binding of a noncleavable substrate analog to the ribozyme (A, B), or formation of domain B, by binding of the strand containing the 3' end of the ribozyme (C). Cleavage assays were performed with a series of substrate 3' deletion variants within helix 1 (A), with a series of substrate 5' deletion variants in helix 2 (B), and with a series of mismatches in helix 3 (C).

marked D and G show that much of domain A can be removed without diminishing the cleavage rate.

Analogous experiments were done to determine how the activity of ribozymes bearing deletions in helices 1 and 2 would be affected by mismatches in helix 3 (Fig. 3B). In all cases, the introduction of a mismatch at the junction of helices 2 and 3 (mismatch 1) had very small effects on cleavage rate, while the interior mismatches were more highly inhibitory.

Finally, we used the same approach to test the activity of constructs containing truncations to helices 2 and 3 that alter the register between domains A and B. Cleavage activity was only observed with a symmetrical construct having two base pairs in each domain (Fig. 4A), and activity was quite low ($6 \times 10^{-4} \text{ min}^{-1}$). This strongly suggests that the register between the two domains is crucial, in that it is likely to determine whether or not key interactions between the domains (e.g., the G + 1 · C25 base pair) can be formed.

Cleavage activity of a construct lacking helix 1

Analysis of the preceding data, particularly the combination of deletions, led us to the design of a novel minimal motif

for the hairpin ribozyme, in which helix 1 is removed (Fig. 4B). In this construct, the 5' end of the truncated ribozyme is covalently linked to the 3' end of the truncated substrate, forming a domain A consisting entirely of a hairpin-loop structure containing sequences from loop A and helix 2.

Remarkably, this construct cleaves at a rate that is only 2.5-fold slower than the rate of the trans-cleaving or self-cleaving unmodified ribozyme (0.04 min^{-1} , Fig. 4), supporting the conclusion that sequences of helix 1 have no essential function in either assembly of the active tertiary structure, or in catalysis itself. To ensure that this highly modified ribozyme was an accurate reporter of active site architecture, we examined the effect of combinations of mutations at substrate position +1 and ribozyme position 25 (Fig. 4C). Activity was observed only with the Watson-Crick combinations G + 1 · C25 and A + 1 · U25. This result and the relative activity of the molecules are entirely consistent with the effects of the same base substitutions in the standard ribozyme construct (Pinard et al. 1999b).

To further reduce the size of this self-cleaving construct, we made sequential deletions of helix 4 distal to loop B. Results showed that helix 4 can be reduced to four base

FIGURE 3. Combinations of deletions that retain high levels of catalytic activity. Simultaneous deletions in the substrate and in the substrate binding strand were performed and cleavage rates were determined under saturating and single turnover conditions. The cleavage rate for the reference molecule (WT [wild type], Fig. 1) is indicated in gray. The rate for each combination is labeled with a letter corresponding to the schematic representations *beneath* each panel. Base pairs in helices were indicated with a short line only when it was modified from the original sequence. (A) Cleavage rates of constructs where the extremities of the helices bounding loop A were shortened systematically. (B) Cleavage rates obtained with one (mm 1), two (mm 2), or three (mm 3) mismatches in helix 3 starting from the junction between helices 2 and 3, and proceeding away from the junction. The combined effects of mismatches in helix 3 and deletions in domain A are also shown. Wild-type rate is indicated in white.

pairs while retaining high levels of activity, but that activity was significantly compromised by further deletions (data not shown).

Trans-cleavage reaction with a hairpin-loop substrate

Previous studies have shown that the two domains of the ribozyme can be physically separated, and can then reassociate into an active complex in solution under conditions of relatively high concentrations of magnesium ions or cobalt (III) hexammine (Butcher et al. 1995; Hampel et al. 1998). Consequently, we separated our new, minimal self-cleaving construct into the component domains, and examined their ability to reassociate *in trans* to form a reactive complex (Fig. 5). A reaction was observed to give the expected products, and at high RNA concentrations the reaction rate (0.04 min⁻¹) was equivalent to that of the single-chain molecule. The apparent K_M for forming the reactive complex in was 3.8 μ M, approximately 10-fold higher affinity than the value that was obtained for the full domain A in magnesium (Butcher et al. 1995), but similar to the binding affinity of domain A in cobalt hexaammine (K. Hampel, unpubl.).

Molecular modeling of the minimal substrate

To determine the likely structural consequences of removing helix 1 and joining ribozyme and substrate sequences,

we conducted a molecular modeling exercise, using MC-SYM software (Materials and Methods; Major et al. 1991; Major and Griffey 2001). Results showed that the truncated ribozyme adopts a conformation that is nearly identical to that generated for the complete molecule (Fig. 6).

DISCUSSION

High-resolution structural data provide snapshots of RNA conformation in the undocked and docked states of the hairpin ribozyme–substrate complex (Cai and Tinoco 1996; Butcher et al. 1999; Rupert and Ferré-D'Amaré 2001), and show that docking and undocking are accompanied by major conformational changes in both structural domains. However, little is known about the details of those changes, or about additional conformational changes that may take place during catalysis. The studies described in this paper represent an effort to facilitate such a detailed conformational analysis by defining a minimal RNA that is capable of native folding and reactions.

Our results show that helix 1, although clearly important for substrate binding, does not have an essential function in either tertiary structure formation or catalysis. Deletions in helix 1 reduce substrate binding affinity, consistent with previous results (Donahue et al. 2000). The finding that helix 1 is important for formation of secondary structure,

FIGURE 4. Structure and activity of truncated ribozyme-substrate complexes. (A, *Top*) Sequence and secondary structure of the two-piece hairpin ribozyme construct containing only two base pairs in helix 2 and helix 3 (H22-H32). (*Bottom*) Electrophoretic analysis of the cleavage reaction performed with the H22-H32 construct. (B, *Top*) Sequence and secondary structure of the two-piece hairpin ribozyme construct derived from the deletion analysis and lacking helix one (Rz-substrate-H1). (*Bottom*) Electrophoretic analysis of the cleavage reaction performed with this construct. This complex cleaves at a rate only 2.5-fold lower than that of the wild-type construct. Incomplete cleavage may result from conformational heterogeneity, chemical heterogeneity (e.g., incomplete deprotection), or both. (C) Electrophoretic analysis using the Rz-substrate-H1 construct showing that the cleavage reaction was observed only when the +1:25 interdomain base pair was present. In each panel, cleaved and uncleaved products are indicated by arrows.

but not tertiary structure, is consistent with the results of biophysical and crystallographic studies, which show that helices 1 and 4 approach one another but do not form

functional contacts during tertiary structure formation (Walter et al. 1998, 1999; Porschke et al. 1999; Zhao et al. 2000; Rupert and Ferré-D'Amaré 2001). The modest in-

FIGURE 5. Cleavage assays using minimal separated domains. Sequence and secondary structure of the minimal A and B domains developed in this study (top left). Determination of the apparent K_M of the reaction using cleavage rates (k_{obs}) at various appropriate concentrations of the minimal B domain (top right). Cleaved and uncleaved products are indicated by arrows on the right of the autoradiogram (bottom).

crease in cleavage rate resulting from partial deletion of helix 1 might be attributable to a decrease in product reassociation and subsequent ligation (Fedor 1999).

Results of our analysis of helices 2 and 3, together with a previous study (Pinard et al. 2001b), strongly suggest that docking of ribozymes containing a two-way helical junction is accompanied by fraying of base pairs at the helix 2–3 interface. These data are consistent with the absence of a requirement for the formation of strong base pairs at these positions, as shown by *in vitro* selection (Joseph et al. 1993) and chemical modification analysis (Butcher and Burke 1994). The observed increase in cleavage rates resulting from the introduction of mismatches or short deletions at the interface can be interpreted as resulting from increased conformational flexibility when one or more of the base pairs in helix 2 (–3 · 13, –4 · 14) or helix 3 (15 · 49, 16 · 48) are destabilized. These alterations at the interface of the two helices might also decrease the tendency of the ribozyme domains to form an inactive, coaxially stacked conformation (Esteban et al. 1998). Alterations at the helical interface that stabilize coaxial stacking, such as deletion of the unpaired terminal adenosine, decrease the cleavage rate of the ribozyme (Walter et al. 1998), whereas short deletions at the interface may decrease the stability of coaxial stacking and increase the activity of the population of molecules.

Together, the results of our activity assays, mutational analysis of the G + 1 · C25 base pair, and molecular modeling all provide strong evidence that the truncated ribozyme and substrate form an active complex whose tertiary structure

and catalytic activity are very similar to the structure and activity of the unmodified ribozyme–substrate complex. With these results in hand, we believe that the truncated ribozymes and substrates will be very useful tools for a detailed analysis of interdomain interactions, and for elucidating the conformational changes that accompany docking and catalysis.

MATERIALS And METHODS

RNA preparation

All oligoribonucleotides were generated by solid-phase synthesis using standard RNA phosphoramidite chemistry. Reagents were obtained from Glen Research. Following deprotection, RNA was purified by denaturing gel electrophoresis and reverse-phase HPLC, as previously described (Pinard et al. 2001a).

Ribozyme cleavage assays

All reactions were performed in 50 mM Tris-HCl (pH 7.5), 12 mM MgCl_2 at 25°C. Ribozymes were preincubated at 37°C for 15 min

FIGURE 6. Three-dimensional representations of the ribozyme–substrate complex. The model in A was generated by MC-SYM, and subjected to energy minimization, both as described in Materials and Methods. Stereoviews are displayed, showing the ribozyme–substrate lacking helix one (A) and domain A from the crystal structure (Rupert and Ferré-D’Amaré 2001) (B). The substrate is depicted in red and the substrate binding strand in blue. The cleavage site is indicated by an arrow. Residues 24–26, and 36–38 from domain B are depicted in green and yellow, respectively. The structure of the region surrounding the cleavage site is nearly identical in each construct. Note that there are differences in the sequence between our minimal construct and the crystallographic construct.

in reaction buffer, then solutions were equilibrated at 25°C for 10 min. Cleavage reactions were initiated by adding 5'-³²P-end-labeled substrates (1–2 nM), previously equilibrated in cleavage buffer. At indicated time points, 2 µL aliquots were withdrawn, and quenched with 18 µL loading solution (97% formamide, 15 mM EDTA). To ensure that reactions were initiated with all of the substrate bound to ribozyme, cleavage reactions were always carried out using three different concentrations of ribozyme, with a molar excess of ribozyme to substrate of at least 100. Labeled substrate was present at approximately 1 nM. Ribozyme concentrations ranged from 100 nM to 10 µM. Cleavage reactions were followed long enough to obtain at least 80% cleavage of substrate. Cleavage rates were determined by nonlinear regression analysis using Microcal Origin software. Cleavage kinetics typically displayed single-exponential curve fits. The standard error between fitted curves was generally less than 10%.

Binding and Michaelis constants

Gel mobility-shift assays were used to estimate dissociation constants. Ribozymes were assembled from equimolar quantities of the two or three component RNA strands, and were then incubated with a range of concentrations of the ³²P-labeled strand for which the dissociation constant was to be determined. Homogeneity of the assembled ribozyme was monitored by labeling one of the component strands to low specific activity. Noncleavable substrate contained a 2'-deoxyribose at the cleavage site (dA⁻¹), a variant shown not to interfere with binding (Hampel et al. 1998). Complex formation was analyzed by autoradiography following electrophoresis through nondenaturing polyacrylamide gels. Kinetic experiments were used to estimate K_M values, obtained by curve-fitting of observed cleavage rates (k_{obs}) as a function of the concentration of domain B.

Molecular modeling

Models of the ribozyme–substrate binding domain and the catalytic core were generated using the constraint-satisfaction program MC-SYM (Major et al. 1991; Major and Griffey 2001). The structural constraints used to generate the MC-SYM script were derived from data generated in this work, from previously published topographic work (Pinard et al. 1999b, 2001b), and crystallographic data (Rupert and Ferré-D'Amaré, 2001). The scripts are available at http://www-lbit.iro.umontreal.ca/McSym_Repository/. A-form RNA was assumed for all Watson-Crick helices, and all possible conformations were tested for the sugar pucker models and glycosyl angles for all nucleotides in loop A and for residues 24 to 26, and 36 to 38 of loop B. Solutions that were similar ($\leq 2\text{Å}$ RMSD) were combined by MC-SYM. Resulting structures were refined through molecular mechanics calculations performed by the molecular simulation program Sander, from the Amber 4.1 suite of programs (Pearlman et al. 1995) using the Amber 94 force field. All 1–4 electrostatic interactions were set to a factor of 1.2, and the distance-dependent dielectric model ($\epsilon = 4R_{ij}$) for the Coulombic representation of electrostatic interactions was used. As a first step, energy minimization was performed using the steepest descent for 100 steps, then the conjugate gradient method was applied until the maximum derivative was less than 0.1 kcal/mole Å. Figures were prepared using MolScript (Kraulis 1991).

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