FUNCTIONAL ANALYSIS OF HAIRPIN RIBOZYME ACTIVE SITE ARCHITECTURE*
Joseph W. Cottrell, Yaroslav I. Kuzmin2, and Martha J. Fedor1
From the Department of Molecular Biology and The Skaggs Institute for Chemical Biology
The Scripps Research Institute, La Jolla, CA 92037

RUNNING TITLE: Functional Analysis of Hairpin Ribozyme Architecture

SUMMARY
The hairpin ribozyme is a small catalytic motif found in plant satellite RNAs where it catalyzes a reversible self-cleavage reaction during processing of replication intermediates. Crystallographic studies of hairpin ribozymes have provided high-resolution views of the RNA functional groups that comprise the active site and stimulated biochemical studies that probed the contributions of nucleobase functional groups to catalytic chemistry. The dramatic loss of activity that results from perturbation of active site architecture points to the importance of positioning and orientation in catalytic rate acceleration. The current study focuses on the network of noncovalent interactions that align nucleophilic and leaving group oxygens in the orientation required for the S$_\text{N}2$-type reaction mechanism and orient the active site nucleobases near the reactive phosphate to facilitate catalytic chemistry. Nucleotide modifications that alter or eliminate individual hydrogen bonding partners had different effects on the activation barrier to catalysis, the stability of ribozyme complexes in the ground state, and the internal equilibrium between cleavage and ligation of bound products. Furthermore, substitution of hydrogen bond donors and acceptors with seemingly equivalent pairs sometimes had very different functional consequences. These biochemical analyses augment high-resolution structural information to provide insights into the functional significance of active site architecture.

The well-characterized structure of the hairpin ribozyme offers a valuable framework for investigating the contributions of individual active site interactions to the activation barrier to catalysis and to the stability of ribozyme complexes in the ground state. The hairpin ribozyme catalyzes a reversible self-cleavage reaction in which nucleophilic attack of a ribose 2'-hydroxyl on an adjacent phosphorus proceeds through a trigonal bipyramidal transition state that leads to the formation of 2',3'-cyclic phosphate and 5'-hydroxyl termini (1). High-resolution crystal structures have been solved for hairpin ribozymes in complexes with a noncleavable substrate analog, cleavage products and a vanadate mimic of the trigonal bipyramidal transition state, making this ribozyme the subject of more detailed structural analyses than virtually any other catalytic RNA (2-6) (Fig. 1).

Hairpin ribozymes have two essential helix-loop-helix domains, A and B, that associate to form the active site (7). High resolution structures reveal a network of stacking and hydrogen bonding interactions within the active site that align the reactive phosphate in the appropriate orientation for an S$_\text{N}2$-type nucleophilic attack mechanism and orient nucleotide base functional groups near the reactive phosphate to facilitate catalytic chemistry (Fig. 1). G+1 is the conserved nucleotide on the 3’ side of the reactive phosphodiester (Fig. 2). Tertiary interactions between G+1 and nucleotides in loop B define the architecture of the active site (2,8). Guanine at the +1 position has no direct contact with the reactive phosphodiester but loss of this nucleobase virtually eliminates catalytic activity, highlighting the significant contribution of active site architecture to catalytic rate enhancement (8-12). Positioning and orientation also play critical roles in the internal equilibrium between cleavage and ligation of bound products. Interdomain junction modifications and changes in reaction conditions that destabilize ribozyme tertiary structures shift the internal equilibrium toward cleavage (13-16). The relationship between tertiary structure stability and the internal equilibrium is consistent with the idea that ligation requires a rigid ribozyme structure to align the 2',3'-cyclic phosphate and 5'-hydroxyl termini, while these reactive groups are fixed by the diester linkage in a cleavage substrate.

Biochemical studies have shown that interactions that seem to be equivalent in hairpin ribozyme crystal structures can make very different energetic contributions. For example, the N6
exocyclic amines of A9 and A38 both appear to donate hydrogen bonds to the same proR₆ nonbridging oxygen of the reactive phosphate (3), but substitution of adenine with purine at position 38 inhibits cleavage activity more than 10⁵ fold (17) while complete elimination of adenine at position 9 through an abasic substitution reduces cleavage activity just 4 fold (18). The dramatic difference in the functional significance of two seemingly identical interactions illustrates the need for direct analyses of structure-function relationships that might be inferred from crystal structures.

Minimal hairpin ribozymes, in which the A and B domains are joined by a two-way helical junction, partition almost equally between an inactive, extended conformation and a functional structure that juxtaposes loops A and B to create the active site (19). Extensive structure-function studies of minimal hairpin ribozymes have been reported previously (1). However, it is now understood that most modifications that disable minimal ribozymes do so by shifting the conformational equilibrium further toward the inactive, extended structure (19-21). In viral satellite RNAs, the A and B domains comprise two arms of a four-way helical junction (7,22). Restoring this natural junction stabilizes the docked structure and ribozymes with a four-way junction retain activity despite unfavorable reaction conditions and loss of individual tertiary interactions (14,16,19,21,23). This discovery makes it possible to examine the effects of nucleotide modifications in the context of the native folded structure. We report a systematic analysis of the active site architecture of a four-way junction ribozyme aimed at comparing the energetic consequences of functional group modifications on the stability of ribozyme complexes in the ground state, the activation barrier to catalysis, and the internal equilibrium between cleavage and ligation of bound products.

**EXPERIMENTAL PROCEDURES**

RNA preparation—RNAs were prepared by T7 RNA polymerase transcription of linearized plasmid templates or by chemical synthesis (Fig. 2, Table 1) (Dharmacon), as described previously (12,14). LR43 is a self-cleaving ribozyme that assembles in the context of a four-way helical junction and has three base pairs in the intermolecular helix, H1, that forms between the 5' and 3' cleavage product RNAs (Fig. 2A). 5'R4 RNA was obtained from LR43 self-cleavage during bacteriophage T7 RNA polymerase transcription of the pTLR43 template. 5'R4 was combined with 3'P3 RNAs obtained through chemical synthesis to measure cleavage kinetics through ligation-chase assays, as described below. LR46 and LR47 variants are four-way junction ribozymes with six or seven base pairs in H1, respectively, which were produced by ligation of 5'R4, the 5' product of LR43 self-cleavage, and 3'P6 or 3'P7 RNAs obtained through chemical synthesis (Fig. 2B). LR46 and LR47 variants were used to measure ligation kinetics and equilibrium dissociation constants for 5'R4-3'P complexes. The pTLR43G+1A,C25U plasmid template encodes the LR43G+1A,C25U self-cleaving ribozyme variant, and was derived from pTLR43 by using QuikChange mutagenesis to replace the guanine at position +1 with adenine and the cytosine at position 25 with uridine. Self-cleavage of the LR43G+1A,C25U ribozyme variant produces 5'R4C25U and 3'P3G+1A product RNAs. The ribozyme RNA, R4, and substrate RNA, S43, which were used for intermolecular cleavage reactions (14) (Fig. 2C), were prepared through T7 RNA polymerase transcription of the pTR4 plasmid template and through chemical synthesis, respectively.

Ribozymes with modifications at positions 36 and 38 were prepared through ligation of synthetic RNAs (Table 1, Fig. 2C). First, LR43AAAB(5'GA3)C+6U and B were phosphorylated in reactions with 1 nmol oligonucleotide, 2 nmol ATP and 5 units T4 polynucleotide kinase in a final volume of 5 µL. After a 30 min incubation at 37°C, the kinase was inactivated at 90°C for 2 min. Next, stoichiometric amounts of LR43AAAB(5'GA3)C+6U, B and A(B’3’GA3)G4A RNAs were annealed in T4 polynucleotide kinase buffer (NEB) by heating to 85°C for 1 min and cooling to 30°C over 4 min. Incubation at 30°C for an additional 20 to 40 min led to nearly complete cleavage of LR43AAAB(5'GA3)C+6U for oligonucleotides with noninhibitory modifications of A38 and G36, producing a 2',3’-cyclic phosphate terminus. Ribozymes with inhibitory modifications (A38dX) were induced to cleave by adding 20 mM isocytosine to the reaction and extending incubation times to 4 hours (17,18). Covalent joining of ribozyme fragments was accomplished by adding 1 mM ATP and 20 units T4 RNA ligase and incubating overnight at 17°C, followed by heat inactivation of the ligase at 90°C for 2 min. Full length ribozyme products were fractionated by denaturing gel electrophoresis, eluted and prepared as sodium salts using DEAE ion exchange chromatography as described previously (14) with a typical yield of 120 pmol of ribozyme per 1 nmol of each of the three oligonucleotide components. Chemical synthesis of oligonucleotides that
correspond to the 3' product of ribozyme cleavage was used to introduce modifications at the G+1 position. Abasic substitutions were introduced as deoxyribonucleotides because abasic ribonucleotides are not yet available commercially.

3' product RNAs were labeled at the 3' terminus by \(^{32}\)P capping (24). Briefly, 75 pmol of cytosine monophosphate (Cp) was combined with 25 pmol of \([\gamma-^{32}\text{P}]/\text{ATP}\) (5,000 Ci/mmole) and 5 units T4 polynucleotide kinase for 1 hr at 37°C to produce \(^{32}\)P Cp. Following heat inactivation of the kinase, the mixture was added to a solution containing 12.5 pmol 3' product RNA, 10% dimethyl sulfoxide, 1 mM ATP, polynucleotide kinase buffer, and 2 units T4 RNA ligase and incubated overnight at 15°C. Products were separated by denaturing polyacrylamide gel electrophoresis, eluted, then desalted by NAP column chromatography (Amersham Biociences) and reduced to a volume of approximately 40 µL by lyophilization.

**Measurement of Equilibrium Dissociation Constants**—Equilibrium dissociation constants for 5'R-3'P complexes, \(K_{d,5'R-3'P}^{app}\), and values for the internal equilibrium between cleavage and ligation of bound products, \(K_{eq}^{int}\), for 5'R-3'P complexes with rapid ligation kinetics were determined from the RNA concentration dependence of observed ligation rates as described (13,14). Briefly, maximum ligation extents, \(F_{max}\), were determined from reactions with varying concentrations of 5'R and 0.1 – 0.25 nM of \(^{32}\)P-labeled 3’P6 or 3’P7 at 15°C in 10 mM MgCl₂, 50 mM NaHepes pH 7.5, 0.1 mM EDTA by computing the fit to equation 1,

\[
FLR_{obs} = F_{active} \times \frac{\left[5'R\right] \times FLR_{max}}{\left(K_{d,5'R-3'P}^{app} + \left[5'R\right]\right)}
\]

where \(FLR_{obs}\) is the fraction of ligated ribozyme at the end of a reaction, \(F_{active}\) is the fraction of active ribozyme complex that is correctly folded and has the necessary 2',3'-cyclic phosphate terminus, and \(K_{d,5'R-3'P}^{app}\) is the apparent equilibrium dissociation constant for the 5'R-3'P complex. \(FLR_{max}\) is the maximum theoretical fraction of ligated ribozyme calculated from the equilibrium between cleavage and ligation rate constants, \(K_{eq}^{int} = k_{lig}/k_{cleav}\), according to

\[
FLR_{max} = K_{eq}^{int}/(K_{eq}^{int} + 1) = k_{lig}/(k_{lig} + k_{cleav}).
\]

Within the same set of experiments, \(F_{active} \times FLR_{max}\) remains constant so that equation 1 reduces to equation 2.

\[
FLR_{obs} = \frac{\left[5'R\right] \times C}{\left(K_{d,5'R-3'P}^{app} + \left[5'R\right]\right)}
\]

\(K_{d,5'R-3'P}^{app}\) is related to the thermodynamic equilibrium dissociation constant, \(K_{d,5'R-3'P}^{eq}\), according to

\[
K_{d,5'R-3'P}^{app} = K_{d,5'R-3'P}^{eq}/(K_{eq}^{int} + 1).
\]

Therefore, \(K_{d,5'R-3'P}^{eq}\) can be calculated from \(K_{d,5'R-3'P}^{app}\) using \(K_{eq}^{int}\) values obtained from the cleavage and ligation rate constants that were measured independently, as described below.

For ribozyme variants with slow ligation kinetics, equilibrium dissociation constants for 5'R-3'P complexes were determined directly from the concentration dependence of complex formation using gel shift assays, as described previously (25). Briefly, 0.1 – 0.25 nM of \(^{32}\)P-labeled 3’P product RNA was incubated with varying concentrations of 5'R for 2 to 4 hours at 15°C in 10 mM MgCl₂, 50 mM NaHepes pH 7.5, 0.1 mM EDTA to allow complex formation to reach equilibrium. Bound and unbound \(^{32}\)P-labeled 3’P were fractionated by electrophoresis in nondenaturing polyacrylamide gels with 15% acrylamide (19:1, acrylamide/bisacrylamide), 10 mM magnesium acetate, 50 mM Tris acetate, pH 7.5 for 12 hours at 35 mA at a constant temperature of 5°C in a vertical slab gel electrophoresis unit (Hoefer SE600). Equilibrium dissociation constants were calculated from the fit to equation 3, where \(F_{complex,obs}\) is

\[
F_{complex,obs} = \frac{\left([LR] + [5'R-3'P]\right)/\left([LR] + [5'R-3'P] + [3'P]\right)}{(K_{d,5'R-3'P}^{eq} + [5'R])]}
\]

Cleavage Kinetics Assays—Cleavage rate constants were determined at 15°C in 10 mM MgCl₂, 50 mM NaHepes pH 7.5, 0.1 mM EDTA, the same conditions used for equilibrium binding assays, from intermolecular cleavage assays with saturating concentrations of ribozyme, as described (18,25,26). Ligation-chase assays were used to monitor self-cleavage rates directly within a fully functional ribozyme created by self-ligation, as described (18). Briefly, 0.1 – 0.25 nM of \(^{32}\)P-labeled 3’P3 was combined with 5’ ribozyme RNA at a concentration approximately equal to the apparent equilibrium dissociation constant for product binding and allowed to undergo ligation to yield a final fraction of ligated ribozyme of at least 20%. To maximize ligation efficiency in reactions with 5’RA38dX, exogenous isocytosine was included at a concentration of 1 mM. Ligation reactions were then diluted 40- and 80-fold into
reaction buffer to initiate cleavage. Aliquots were quenched after various times, fractionated on denaturing gels and quantified by radioanalytic imaging (Molecular Dynamics). Cleavage rate constants were computed from fits to the equation

\[ FP_t = FP_{t=\infty} \times (1 - e^{-k_{\text{cleavage}} t}) + FP_{t=0}, \]

where \[ FP = [3'P]/([LR]+[3'P]) \]. Each reported value represents the mean of two or more independent measurements; reported errors are standard deviations.

3’ product RNA sequences were chosen to form an intermolecular H1 helix with a stability optimized to ensure that product binding affinity was high enough to support adequate ligation extents at experimentally accessible RNA concentrations during a ligation pulse but low enough to ensure that cleavage rates observed following a 40-fold dilution were not complicated by slow product dissociation or product re-binding and ligation. Confirmation that similar cleavage rates were observed following 40 and 80-fold dilutions ensured that no significant product rebinding and ligation occurred during the chase phase. For unmodified RNAs and modified RNAs that form stable complexes (dG+1, mG+1, dA38), we used a 3’ product RNA, 3’P3(+2), that forms an H1 helix with three base pairs. 3’P3(+2) contains a 2-nucleotide 3’ overhang so that 3’ terminal labeling with \( ^{32} \)pCp does not affect H1 stability. For modified RNAs that form less stable complexes (G+1I, G+1(2AP), A38dX) pulse-chase experiments were carried out using 3’P3 RNA, which forms an H1 helix with four base pairs after 3’ end labeling with \( ^{32} \)pCp. In each case, results of experiments with different 3’P RNAs that have different binding affinities were compared to confirm that observed cleavage rates monitored the cleavage step and not slow product dissociation or re-binding and ligation of bound product.

Ligation Kinetics Assays—Ligation kinetics were measured under the same conditions used for cleavage and equilibrium binding assays. 5’R, at concentrations between 500 nM and 4 \( \mu \)M, first was heated to 85°C for 1’ in 50 mM NaHepes pH 7.5, 0.1 mM then slowly cooled to 15°C in the presence of 10 mM MgCl2. \( ^{32} \)pCp-labeled 3’P6 (or 3’P7) was added to a final concentration of 0.1 – 0.25 nM to initiate ligation. Samples were removed at intervals, quenched, fractionated, and quantified as described above. Observed ligation rates were computed from fits to the equation

\[ \text{FLR}_t = \text{FLR}_{t=\infty} \times (1 - e^{-k_{\text{ligation}} \text{obs.} t}) + \text{FLR}_{t=0}, \]

where \[ \text{FLR} = [LR]/([LR]+[3'P]) \]. Comparisons of observed ligation rates and extents in reactions with at least a two-fold difference in 5’R concentrations confirmed that ligation rates were not limited by slow 5’R-3’P complex formation. Ligation rate constants were calculated from the difference between the cleavage rate constant obtained from pulse – chase experiments and the mean observed ligation rate, which reflects the rate of approach to equilibrium between cleavage and ligation and is the sum of ligation and cleavage rate constants, as described previously (13).

RESULTS AND DISCUSSION

The functional consequences of active site modifications were determined from changes in cleavage and ligation rate constants and equilibrium dissociation constants for the complex formed between 5’ ribozyme and 3’ product RNAs, 5’R-3’P, as illustrated by the reaction scheme shown in Fig. 3. Kinetic and equilibrium parameters were measured at pH 7.5 in reactions with 10 mM MgCl2. These conditions are similar to those previously used for analyses of similar hairpin ribozymes (12,14,17,18), except that a temperature of 15°C was chosen, rather than the standard temperature of 25°C, because the lower temperature facilitated comparisons of ribozyme variants with destabilizing modifications. A ligation-chase protocol was used to measure cleavage rates to ensure that cleavage assays monitored the catalytic activity within properly folded 5’R-3’P complexes and were not complicated by slow refolding of misfolded RNAs (13). Changes in cleavage and ligation rate constants reflect the effects of modifications on the magnitude of the activation barrier to cleavage or ligation, \( \Delta G^\ddagger \), while changes in equilibrium dissociation constants reflect effects of modifications on the ground state stability of 5’R-3’P complexes, or \( \Delta G_{5’R-3’P}^{\ddagger} \). It is important to recognize that observed changes in transition state and ground state energies that result from functional group modifications are not a direct measure of the energetic contribution of the bonds formed between specific functional groups because changes in interactions with solvent also contribute to free energy changes (27).

Functional consequences of G+1 modifications—The active site assembles through tertiary interactions between loops A and B (2,20,22,28) (Fig. 1). Crystallographic studies revealed very similar active site structures for a ribozyme complex with vanadate, a mimic of the trigonal bipyramidal transition state in which phosphorus associates with five oxygen atoms, and a ribozyme complex with product RNAs that have 5’ hydroxyl and 2’,3’-cyclic phosphate termini (3) (Fig. 1A, B). In both vanadate and product complexes, G+1...
extrudes from loop A in an unusual syn conformation and stacks between the A38 and A26 nucleobases in loop B. G+1 forms additional hydrogen bonding interactions with loop B nucleotides that include Watson-Crick pairing between G+1 and C25 (8) and hydrogen bonding between N7 of G+1 and the 2′OH of A38 and between the 2′ hydroxyl of G+1 and the exocyclic amine of G36. The absence of significant differences between the two structures in the distances between G+1 functional groups and putative hydrogen bonding partners in loop B suggests that G+1 forms virtually the same hydrogen bonding and stacking interactions in the ground state as in the transition state.

G+1 is the 5′ terminal nucleotide of the 3′ cleavage product RNA (3′P) so changes in ground state interactions with G+1 will be reflected in equilibrium dissociation constants for 3′ product RNA binding (14). The length and sequence of the intermolecular H1 helix that forms between 5′ ribozyme and 3′ product RNAs can vary without loss of catalytic activity (1). Therefore, 3′ product RNAs can be designed to form H1 helices with the optimal number of base pairs to facilitate measurements of cleavage or ligation kinetics or equilibrium dissociation constants for 5′R·3′P complexes (14,15,25) (Fig. 2). The LR43 and LR44 self-cleaving ribozyme variants with three or four base pairs in H1, respectively, were used to measure self-cleavage kinetics (Fig. 2A). Using variants with small H1 helices ensured that product dissociation was much faster than the ligation step so that observed self-cleavage rates were not complicated by slow product dissociation and re-ligation of bound products. LR46 or LR47 variants with six or seven base pairs in H1, respectively, were used to measure ligation kinetics and equilibrium dissociation constants for ribozyme-product complexes (Fig. 2B). Using variants with six or seven base pairs in H1 ensured that $K_d$ values fell into the measurable range, between 0.1 and 1,000 nM, even when 3′ product RNAs contained destabilizing G+1 modifications.

Substitution of G+1 with purine eliminates the possibility of any Watson-Crick hydrogen bonding interactions with C25 but the purine nucleotide retains the ability to form hydrogen bonds with ribose oxygens; N7 of purine is available to accept a hydrogen bond from the 2′ hydroxyl of A38 and the 2′ hydroxyl at the +1 position still could donate a hydrogen bond to N6 of G36 (Fig. 1). Purine at the G+1 position also retains the ability to stack between A38 and A26. Comparison of 5′R complexes with 3′P6 or 3′P6G+1Pu RNAs that both form H1 helices with six base pairs shows that the G+1Pu substitution increased the equilibrium dissociation constant for 3′P binding from a $K_d$ value of about 6 nM for the unmodified 3′P6 RNA to a $K_d$ value of 590 nM for the 3′P6 G+1Pu RNA (Table 2). This 100-fold decrease in $K_d$ values corresponds to a $\Delta G_{5′R·3′P}^{\text{G+1Pu}}$ value of +2.6 kcal/mole. A 5′R complex with 3′P7G+1Pu RNA, which contains the G+1Pu substitution but forms a seventh base pair in the H1 helix, displayed a $K_d$ value of 20 nM. This value is just 3-fold greater than the $K_d$ value measured for the 5′R·3′P6 complex with an unmodified 3′ product RNA that forms an H1 helix with six base pairs. Thus, the +2.6 kcal/mole loss of binding energy that results from the purine substitution of G+1 is close to the +2 kcal/mol increase in free energy that results from the loss of a conventional helical base pair in the H1 helix of 5′R·3′P6 relative to H1 of 5′R·3′P7 when both 3′ product RNAs contain the same G+1Pu modification (compare lines 3 and 4, Table 2). This $\Delta G_{5′R·3′P}^{\text{G+1Pu}}$ value of +2 kcal/mole that results from the loss of a helical base pair subsequently was used to deduce the change in binding energy that can be specifically attributed to a G+1 modification when comparing 5′R·3′P complexes with six or seven base pairs in the H1 helix.

**Functional Analysis of Hairpin Ribozyme Architecture**

Complete deletion of the G+1 nucleotide in 3′P7ΔG+1 RNA and substitution of guanine with adenine in 3′P7G+1A RNAs reduced 5′R·3′P complex stability by virtually the same amount as the G+1Pu substitution, with $\Delta \Delta G_{5′R·3′P}^{\text{G+1A}}$ and 3′P7ΔG+1 and 5′R·3′P+1A complexes of +2.6 and +2.8 kcal/mol, respectively (Table 2). Thus, the capacity of G+1Pu and G+1A nucleotides to form similar stacking interactions as G+1, and the same hydrogen bonding interactions with ribose oxygens, contributes little to product binding affinity in the absence of Watson-Crick hydrogen bonding between the nucleobases at positions +1 and 25. These results confirm evidence from FRET studies of interdomain docking equilibria that individual hydrogen bonds in the G+1 binding pocket act cooperatively to stabilize the functional structure (29). Results of these FRET studies indicated that G+1A and G+1Pu modifications destabilized the docked conformation of a four-way junction ribozyme by just +0.9 and +0.6 kcal/mol, respectively. Thus, the loss of Watson-Crick hydrogen bonding between G+1 and C25 nucleobases destabilized 3′ product binding significantly more than it destabilized interdomain docking.

Of course, no activity was detected when 3′ product RNAs lacked the G+1 nucleotide altogether. G+1A modifications previously were
reported to eliminate all detectable cleavage activity in minimal two-way junction ribozymes and in ribozymes that assemble in the context of a four-way helical junction (21,30). We found that complexes with G+1Pu and G+1A modifications displayed so little ligation activity that ligation pulse chase experiments could not be used to measure cleavage kinetics. Cleavage rate constants could only be approximated from intermolecular cleavage reactions with separate ribozyme and substrate RNAs (Fig. 2C). The G+1Pu and G+1A modifications reduced cleavage rate constants by more than 10\(^3\) fold and reduced ligation rate constants by about 10\(^4\)-fold. This loss of cleavage activity corresponds to increases of about +3.9 and +4.3 kcal/mol in the activation barrier to cleavage for G+1Pu and G+1A modifications, respectively (Table 2). These changes in catalytic activity are significantly greater than the approximately +2.7 kcal/mol loss in product binding affinity that resulted from the same G+1 modifications (Table 2). Thus, interactions with the Watson-Crick hydrogen bonding face of G+1 lower the activation barrier to catalysis more than they stabilize product binding in the ground state.

Restoration of single hydrogen bond donor and acceptor pairs between nucleobases at positions +1 and 25 increased binding affinity by amounts that ranged from +1.5 to +2.2 kcal/mol (Table 2). A single amino-imino hydrogen bond could form between the purine N1 in 3PG+1Pu RNA and N3 of uracil at position 25 in 5RC25U RNA (bond 2 in Fig. 1). The 5RC25U·3PG+1Pu complex was more stable than a complex with just the G+1Pu modification or a complex lacking G+1 completely, displaying a \(\Delta \Delta G_I^{5R\cdot3P}\) value of +1.5 kcal/mol relative to an unmodified complex. The 5RC25U·3PG+1Pu complex also displayed significantly more catalytic activity than complexes that are unable to form any Watson Crick hydrogen bonds between nucleobases at the +1 and 25 positions, with a \(\Delta \Delta G_I\) value of just +1.0 kcal/mol relative to an unmodified ribozyme, corresponding to a 5-fold decrease in the cleavage rate constant.

A single hydrogen bond also could form between the keto oxygen of C25 in unmodified 5R and the exocyclic amine of 3PG+1(2AP) RNA or between the exocyclic amine of G+1 in unmodified 3 product RNA and O2 of uridine in ribozyme with a C25U mutation (bond 3 in Fig. 1). 5R·3PG+1(2AP) and 5RC25U·3P complexes with the capacity to form this single N2:O2 hydrogen bond displayed \(K_d^{5R\cdot3P}\) values that were more than 25-fold higher than \(K_d^{5R\cdot3P}\) values of an unmodified complex, with \(\Delta \Delta G_I^{5R\cdot3P}\) values of +2.2 and +2.0 kcal/mol, respectively. Thus, the 5R·3PG+1(2AP) and 5RC25U·3P complexes that were able to form bond 3 were somewhat less stable than the 5RC25U·3PG+1Pu complex that was able to form bond 2. They also displayed somewhat lower cleavage activity. Cleavage rate constants for 5R·3PG+1(2AP) and 5RC25U·3P complexes were reduced by about 15-fold relative to an unmodified ribozyme complex, corresponding to a \(\Delta \Delta G_I\) value of +1.5 kcal/mol (Table 2).

Previously, a minimal ribozyme with a G+1(2AP) substitution was reported to display the same cleavage rate constant as an unmodified ribozyme (30). Thus, it appears that the potential to form a hydrogen bond between the N2 exocyclic amine of a purine nucleobase and the O2 keto oxygen of a pyrimidine lowers the activation barrier to catalysis by somewhat less than it stabilizes product binding in the ground state. A 2-aminopurine substitution for G+1 destabilized the docked conformation of a four-way junction ribozyme by just +0.4 kcal/mol (29), suggesting that the potential to form the pyrimidine O2:purine N2 bond also contributes less to the stability of interdomain docking than to product binding affinity.

A C25U substitution reduced cleavage rate constants by just 15-fold, but reduced ligation rate constants almost 500-fold, shifting the internal equilibrium between cleavage and ligation of bound products, \(K_{eq}\), from 36 in the unmodified complex to about 1.2 in the 5RC25U·3P complex (Table 2). Complexes with a single G+1(2AP) substitution also retained less ligation activity than the unmodified ribozyme, displaying a \(K_{eq}\) value of 2. Shifts in the internal equilibrium toward cleavage were observed for virtually all active site modifications although the magnitudes of the shifts varied, as described below.

The 5RC25U·3PG+1(2AP) complex, which has the potential to form two Watson-Crick hydrogen bonds, (bonds 2 and 3, Fig. 1) showed little change in complex stability or in catalytic activity relative to the unmodified ribozyme even though it lacks the pyrimidine N4-purine O6 hydrogen bond (bond 1, Fig. 1). The 5RC25U·3PG+1A complex also has the potential to form two hydrogen bonds (bonds 1 and 2, Fig. 1) in an A:U Watson Crick pair that replaces the G:C pair in the unmodified ribozyme. This complex was about 10-fold less stable than an unmodified complex, corresponding to a \(\Delta \Delta G_I^{5R\cdot3P}\) value of +1.3 kcal/mol. Thus, the loss of stability due to substitution of the A+1:U25 tertiary interaction for the normal G+1:U25 pair is
The 5'R-3'PG+1I complex has the capacity to form the same purine N1:pyrimidine N3 and purine N6:pyrimidine O2 hydrogen bonds as the 5'R25U-3'PG+1A complex (bonds 1 and 2, Fig. 1). However, with a ∆ΔΔG^{3R·3P} value of +2.45 kcal/mol, the 5'R-3'PG+1I complex was only slightly more stable than a 5'R-3'PG+1Pu complex that lacks the capacity to form any hydrogen bonds between nucleotide bases at positions +1 and 25 (Table 2). Despite the 10-fold difference in K_{eq}^{3R·3P} values between 5'R25U-3'PG+1A complexes and 5'R-3'PG+1I complexes, both complexes displayed similar cleavage rate constants of about 0.04 min⁻¹. This rate constant is just 6-fold below the cleavage rate constant of 0.24 min⁻¹ measured for an unmodified ribozyme and more than 10⁴-fold greater than the cleavage rate constant of about 2.7 x 10⁻⁴ min⁻¹ measured for the R4-S4A·G+1Pu complex (Table 2). These results contrast with previous reports that a G+1I substitution eliminates all detectable cleavage activity in the context of a minimal hairpin ribozyme (10,30). Our results show that inosine at the +1 position is nearly as effective as guanine in lowering the activation barrier to catalysis, despite the loss of the pyrimidine O2: purine N6 hydrogen bond, when ribozyme tertiary structure is stabilized in the context of a 4-way helical junction.

This comparison of ∆ΔΔG and ∆ΔΔG^{3R·3P} values for 5'R25U-3'PG+1A and 5'R-3'PG+1I complexes indicates that modifications of hydrogen bonding partners that appear to make similar contributions to active site architecture can have very different effects on catalysis and ground state stability. In particular, the interaction between the amidine group of C25 and inosine at the +1 position (bonds 1 and 2, in Fig. 1) contributes little to 3' product RNA binding affinity but significantly lowers the activation barrier to catalysis. Uracil at position 25 also has the potential to form hydrogen bonds 1 and 2 with adenine at the +1 position in 3'PG+1A RNA, but with the hydrogen bonding polarity reversed. However, the U25:A+1 pair seems to stabilize product binding as much as it lowers the activation barrier to catalysis. These functional differences between C25:I+1 and U25:A+1 pairs would not be predicted from inspection of the structure alone.

The ligation rate constant for the 5'R-3'PG+1I complex was reduced by just 40-fold. With a 6-fold decrease in the cleavage rate constant, the internal equilibrium continued to favor ligation relative to cleavage by 5-fold. Thus, the internal equilibrium continued to favor ligation for each of the modified ribozyme complexes that retain the ability to form two hydrogen bonds between nucleobases at positions 25 and +1 – 5'R-3'PG+1I, 5'R25U-3'PG+1A and 5'R25U-3'PG+1(2AP) – with K_{eq}^{int} values between 5 and 20 (Table 2, Fig. 1). Each of the modified ribozyme complexes that retain just a single hydrogen bonding interaction – 5'R25U-3'PG+1Pu, 5'R25U-G+1, and 5'R-3'G+1(2AP) – displayed a smaller preference for ligation, with K_{eq}^{int} values between 1.2 and 2. Both complexes that lack any potential for interdomain Watson Crick hydrogen bonding – 5'R-3'G+1Pu and 5'R-3'G+1A – displayed K_{eq}^{int} values below 1. Thus, K_{eq}^{int} values for modified ribozyme complexes correlated very well with the number of Watson Crick hydrogen bonding interactions that could form between nucleobases at positions +1 and 25.

This relationship between the internal equilibrium and the capacity for interdomain Watson Crick hydrogen bonding is consistent with earlier evidence that tertiary structure stability is a critical determinant of the hairpin ribozyme proficiency as an RNA ligase. Previous work showed that low temperatures and high cation valency and concentration, all features that tend to stabilize RNA structures, shift the balance between cleavage and ligation of bound products in favor of ligation (13,16). Likewise, hairpin ribozymes that assemble in the context of a four-way helical junction display enhanced tertiary structure stability and much higher ligation activity relative to minimal ribozymes with a bulged or two-way helical junction linkage between the A and B domains (14,19).

Recent biochemical studies of an extended form of the hammerhead ribozyme revealed a similar relationship between tertiary structure stability and ligation proficiency (33). In the natural form of the hammerhead ribozyme, an interdomain loop-loop interaction, which is absent from minimal hammerhead ribozymes, stabilizes the functional tertiary structure (34-36). While the internal equilibrium favors cleavage over ligation by more than 100-fold for minimal hammerhead ribozymes (37), ligation rate constants increased about 2,000-fold in reactions catalyzed by the extended form of the hammerhead ribozyme to give a K_{eq}^{int} value close to 1(33).
A likely explanation for the observation that tertiary structure stability promotes ligation more than cleavage is that ligation depends on active site structure for precise alignment of the 2',3'-cyclic phosphate and 5' hydroxyl termini that are to undergo ligation while the same functional groups are fixed relative to each other by the covalent diester linkage in cleavage substrates. According to this view, multiple hydrogen bonds between G+1 and C25 promote ligation in the hairpin ribozyme not only by stabilizing interdomain interactions but also by constraining the propeller twist of the paired nucleobases in the optimal geometry to fix the 2',3'-cyclic phosphate and 5' hydroxyl termini in the optimal alignment for ligation.

**Nucleobase-ribose interactions**—The network of interactions in the G+1 binding pocket also includes hydrogen bonds between nucleotide bases and ribose oxygens (Fig. 1) (2). The 5'RG36I-3'P and 5'R-3'PaG+1 complexes both lack the potential to form a hydrogen bond between the 2' hydroxyl of G+1 and the N6 exocyclic amine of G36 (bond 5, Fig. 1). Both of these complexes displayed almost the same catalytic rate constants as unmodified complexes. $k_{\text{ligation}}$ values fell by just 2-fold and $k_{\text{cleavage}}$ values even increased slightly (Table 3). $K_d$ values for 5'R-3'PaG+1 and 5'RG36I-3'P complexes also were relatively unperturbed, increasing by just 2- and 4-fold, respectively. The $\Delta G^\circ$ values for 5'R-3'PaG+1 and 5'RG36I-3'P complexes also are consistent with a previous report that a dG+1 modification destabilized the docked conformation of a four-way junction ribozyme by just +0.5 kcal/mol (29).

A second ribose hydroxyl-nucleobase hydrogen bond forms between N7 of G+1 and the 2' hydroxyl of A38 (bond 4, Fig. 1). A deoxyadenosine substitution for A38 had large effects on cleavage activity of minimal ribozymes, reducing the cleavage rate constant by 50-fold (40,41). We observed smaller effects in the context of a four-way junction ribozyme; a deoxynucleotide substitution of A38 reduced cleavage and ligation rate constants by 2- and 12-fold, respectively. The 6-fold shift in the internal equilibrium in favor of cleavage could reflect a modest contribution of this hydrogen bond to aligning cleavage product termini for ligation. Loss of the 2' hydroxyl of A38 had no significant effect on 3' product binding affinity (Table 3).

$G+1$ stacking interactions—$G+1$ stacks between A38 and A26 nucleobases in the loop B binding pocket (Fig. 1A, B). The exocyclic N6 and ring N1 functional groups of A38 interact directly with the 5' oxygen leaving group and nonbridging oxygens of the reactive phosphodiester and A38 appears to participate directly in catalytic chemistry (3,17). An abasic deoxynucleotide substitution of A38 reduced cleavage and ligation rate constants by 14,000-fold and 370,000-fold, respectively (18), corresponding to a $\Delta G^\circ$ value of +5.4 kcal/mol. The significant decrease in catalytic activity associated with loss of the A38 nucleobase is consistent with a major role in catalysis. However, almost full activity could be restored to an abasic ribozyme variant lacking A38 when certain nucleobase analogs were provided in solution (18), suggesting that the active site structure remains sufficiently intact to allow specific nucleobase recognition and binding in a cavity left by the A38 nucleobase deletion. Each of the exogenous nucleobases that was able to restore catalytic activity to abasic variants that lack A38 – isocytosine, 2,2-aminopyridine, 3-methyladenine, and 2,6-diaminopurine – is a planar heterocycle with the amidine functional group that corresponds to the Watson-Crick hydrogen bonding face of adenine. Thus, exogenous nucleobase rescue experiments suggest that that restoration of catalytic activity to the abasic variant occurs through exogenous nucleobase binding in the cavity left by the adenine deletion (17). Strikingly, the purine nucleobase, without an amidine functional group, displayed the same affinity for an abasic ribozyme lacking A38 as the nucleobases with amidine groups that were capable of rescue but purine was unable to restore catalytic activity. Competitive inhibition of exogenous nucleobase rescue by purine argues that interactions with the amidine group of A38 significantly lower the energy barrier in the transition state but make no detectable contribution to ground state stability.

Comparison of the effects of an abasic substitution on catalysis and on ribozyme-product complex stability supports the interpretation that interactions with the amidine group of A38 are more important in the transition state than in the ground state. In contrast to the major contribution of A38 to lowering the activation barrier to catalysis, loss of A38 had only minor effects on stability of the ribozyme-product complex in the ground state. An abasic substitution of A38 reduced 3' product RNA binding affinity by 15-fold, corresponding to a $\Delta G^\circ$ value for the 5'RA38dX-3'P complex of just +1.55 kcal/mol.
The final feature of active site architecture that we evaluated is the nature of the sugar puckers of the nucleotides flanking the reactive phosphate. Ribonucleosides typically adopt a C3'-endo sugar pucker that places the electronegative 3' hydroxyl in the preferred axial orientation (42). However, C2'-endo puckers, which are more characteristic of deoxynucleobases, were observed in the crystal structure at the A-1 and G+1 positions (2). These unusual puckers define the trajectory of the phosphodiester backbone at the reactive phosphate, fixing the adjacent 2' hydroxyl and 5' oxygens in the in-line geometry that is consistent with the Sₜ₂⁻type reaction mechanism.

A deoxyguanosine substitution for G+1 eliminates hydrogen bond donation by the 2' hydroxyl to the keto oxygen of G36 but favors the appropriate C2'-endo conformation. As mentioned above, loss of the 2' hydroxyl from G+1 had very small effects on product binding affinity. Small effects of the deoxynucleotide substitution for G+1 on cleavage and ligation rate constants combined to make the internal equilibrium slightly less favorable for ligation by about 3-fold. The 2'-OCH₃ modification of G+1 reduced 5'R-3'PmG+1 complex stability somewhat more, about 5 fold, and reduced cleavage and ligation rate constants by 2- and 7-fold, respectively. The larger effects of the 2'-OCH₃ modification relative to the 2'-deoxy modification might be explained by different effects on the G+1 sugar pucker. The 2'-OCH₃ modification stabilizes the typical ribonucleoside C3'-endo pucker while the ribose at the G+1 position adopts the C2'-endo pucker characteristic of deoxynucleosides (2,42,43).

**CONCLUSION**

Modifications that changed the potential to form specific interactions that were inferred from crystal structures had different effects on the activation barrier to catalysis, the stability of ribozyme complexes in the ground state, and on the internal equilibrium between cleavage and ligation. All modifications of the G+1 binding pocket inhibited ligation more than cleavage, shifting the internal equilibrium between cleavage and ligation, K_{eq}^{\text{eq}} toward cleavage. These results are consistent with previous evidence that tertiary structure stability is the major determinant of the balance between cleavage and ligation. A striking example of differential effects of active site modifications on ground state stability and catalysis was seen with deletion of an active site adenine, A38, which increased the activation barrier to catalysis by more than +5 kcal/mol but reduced the ground state stability of the ribozyme product complex by just +1.6 kcal/mol. Finally, seemingly equivalent functional group substitutions sometimes had very different functional consequences, exemplified by the significant differences in product binding affinity that were seen for two modified ribozyme complexes with the potential to form the same interdomain hydrogen bonds. These quantitative functional studies complement previous analyses of the contributions of G+1 interactions to tertiary structure stability using FRET and provide one of the most detailed views yet of structure-function relationships that contribute to positioning and orientation within a ribozyme active site.

**REFERENCES**

1. Fedor, M. J. (2000) *J. Mol. Biol.* 297(2), 269-291
2. Rupert, P. B., and Ferré-D’Amare, A. R. (2001) *Nature* 410(6830), 780-786
3. Rupert, P. B., Massey, A. P., Sigurdsson, S. T., and Ferré-D’Amare, A. R. (2002) *Science* 298(5597), 1421-1424
4. Alam, S., Grum-Tokars, V., Krucinska, J., Kundracik, M. L., and Wedekind, J. E. (2005) *Biochemistry* 44(44), 14396-14408
5. Salter, J., Krucinska, J., Alam, S., Grum-Tokars, V., and Wedekind, J. E. (2006) *Biochemistry* 45(3), 686-700
6. Fedor, M. J., and Williamson, J. R. (2005) *Nat. Rev. Mol. Cell Biol.* 6(5), 399-412
7. Hampel, A., and Tritz, R. (1989) *Biochemistry* 28(12), 4929-4933
8. Pinard, R., Lambert, D., Walter, N. G., Heekman, J. E., Major, F., and Burke, J. M. (1999) *Biochemistry* 38(49), 16035-16039
9. Page, M. I., and Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. USA* 68(8), 1678-1683
10. Grasby, J. A., Mersmann, K., Singh, M., and Gait, M. J. (1995) Biochemistry 34(12), 4068-4076
11. Shippy, R., Siwkowski, A., and Hampel, A. (1998) Biochemistry 37(2), 564-570
12. Lebruska, L. L., Kuzmine, I. I., and Fedor, M. J. (2002) Chem. Biol. 9(4), 465-473
13. Nesbitt, S. M., Erlicher, H. A., and Fedor, M. J. (1999) J. Mol. Biol. 286(4), 1009-1024
14. Fedor, M. J. (1999) Biochemistry 38(34), 11040-11050
15. Donahue, C. P., Yadava, R. S., Nesbitt, S. M., and Fedor, M. J. (2000) J. Mol. Biol. 295(3), 693-707
16. Yadava, R. S., Choi, A. J., Lebruska, L. L., and Fedor, M. J. (2001) J. Mol. Biol. 309(4), 893-902
17. Kuzmin, Y. I., DaCosta, C. P., Cottrell, J., and Fedor, M. J. (2005) J. Mol. Biol. 349, 989-1010
18. Kuzmin, Y. I., Da Costa, C. P., and Fedor, M. J. (2004) J. Mol. Biol. 340(2), 233-251
19. Walter, N. G., Burke, J. M., and Millar, D. P. (1999) Nat. Struct. Biol. 6(6), 544-549
20. Walter, N. G., Hampel, K. J., Brown, K. M., and Burke, J. M. (1998) EMBO J. 17(8), 2378-2391
21. Zhao, Z., Wilson, T., Maxwell, K., and Lilley, D. M. (2000) RNA 6(12), 1833-1846
22. Murchie, A. I., Thomson, J. B., Walter, F., and Lilley, D. M. (1998) Mol. Cell 1(6), 873-881
23. Klostermeier, D., and Millar, D. P. (2001) Biochemistry 40(37), 11211-11218.
24. England, T. E., and Uhlenbeck, O. C. (1978) Biochemistry 17(11), 2069-2076
25. Hegg, L. A., and Fedor, M. J. (1995) Biochemistry 34(48), 15813-15828
26. Fedor, M. J. (2004) Methods Mol. Biol. 252, 19-32
27. Fersht, A. R. (1988) Biochemistry 27(5), 1577-1580
28. Wilson, T. J., Zhao, Z. Y., Maxwell, K., Kontogiannis, L., and Lilley, D. M. (2001) Biochemistry 40(7), 2291-2302
29. Klostermeier, D., and Millar, D. P. (2002) Biochemistry 41(48), 14095-14102
30. Chowrira, B. M., Berzal-Herranz, A., and Burke, J. M. (1991) Nature 354(6351), 320-322
31. Freier, S., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., and Turner, D. H. (1986) Proc. Natl. Acad. Sci. USA 83(24), 9373-9377
32. Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. (1999) J. Mol. Biol. 288(5), 911-940
33. Canny, M. D., Jucker, F. M., and Pardi, A. (2007) Biochemistry
34. De la Pena, M., Gago, S., and Flores, R. (2003) EMBO J. 22(20), 5561-5570
35. Khvorova, A., Lescoute, A., Westhof, E., and Jayasena, S. D. (2003) Nat. Struct. Biol. 10(9), 708-712
36. Martick, M., and Scott, W. G. (2006) Cell 126(2), 309-320
37. Hertel, K. J., Herschlag, D., and Uhlenbeck, O. C. (1994) Biochemistry 33(11), 3374-3385
38. Pyle, A. M., and Cech, T. R. (1991) Nature 350(6319), 628-631
39. Silverman, S. K., and Cech, T. R. (1999) Biochemistry 38(27), 8691-8702
40. Schmidt, S., Beigelman, L., Karpeisky, A., Usman, N., Sorensen, U. S., and Gait, M. J. (1996) Nucleic Acids Res. 24(4), 573-581
41. Ryder, S. P., and Strobel, S. A. (1999) J. Mol. Biol. 291(2), 295-311
42. Saenger, W. (1984) Principles of Nucleic Acid Structure, Springer-Verlag, New York
43. Lubini, P., Zurcher, W., and Egli, M. (1994) Chem. Biol. 1(1), 39-45

FOOTNOTES

*This work was supported by NIH grant RO1 GM046422 (to M.J.F.).
1To whom correspondence may be addressed: Department of Molecular Biology and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, MB35, La Jolla, CA 92037, USA, Tel.: (858) 784-2770; Fax: (858) 784-2779; E-mail: mfedor@scripps.edu
2Current address: Department of Agricultural Produce Processing and Technology, Novgorod State University, Novgorod, Russia 173000.
3The abbreviations used are: FRET, fluorescence resonance energy transfer; LR, ligated ribozyme; 5'R, 5' product of ribozyme cleavage; 3'P, 3' product of ribozyme cleavage; Pu, purine; I, inosine; mG, 2'-Omethyl guanosine; 2AP, 2-aminopurine; dX, abasic deoxyribonucleotide.
FIGURE LEGENDS

FIGURE 1. Network of tertiary interactions formed with G+1. A. Three dimensional structure of the G+1 binding pocket in a ribozyme complex with a vanadate mimic of the transition state prepared from the coordinates described by Rupert et al. (3) (PDB entry 1M5O) with the vanadate indicated in yellow. B. Three dimensional structure of the G+1 binding pocket in a ribozyme complex with cleavage product RNA prepared from the coordinates described by Rupert et al. (3) (PDB entry 1M5V) with the 2',3'-cyclic phosphate terminus of the 5' cleavage product instead of the vanadate transition state mimic. To help distinguish loop A and loop B residues, G+1 and A-1 nucleotides in loop A are colored light blue and carbon atoms of C25, G36 and A38 nucleotides in loop B are colored green. Tertiary interactions between the essential Loop A and B domains of the hairpin ribozyme define hairpin ribozyme active site architecture and fix the reacting groups in the in-line orientation appropriate for the $S_N$2-type reaction mechanism. The interdomain interface is created by the extrusion of the G+1 nucleotide from loop A into a binding pocket in loop B. C. Diagram of interdomain hydrogen bonding interactions formed with the G+1 nucleotide. Modifications of nucleotides that donate or accept interdomain hydrogen bonds were designed to probe their functional significance.

FIGURE 2. Hairpin ribozyme variants. The natural form of the hairpin ribozyme consists of six helical regions, H1 through H6, and two unpaired loops, A and B. The arrow marks the reactive phosphodiester in loop A. The two essential H1-loop A-H2 and H3-loop B-H4 elements associate noncoaxially within the folded tertiary structure to create the active site. A. The ribozyme variant, LR43, is a self-cleaving ribozyme that assembles in the context of a four-way helical junction and has three base pairs in the intermolecular H1 helix that forms between 5'R4, the 5' product of self-cleavage, and 3'P3, the 3' product of LR43 self-cleavage. LR43 was used to measure effects of modifications on self-cleavage kinetics. B. LR46 is a ribozyme variant with six pair bases in the intermolecular H1 helix that was used to measure effects of modifications on ligation kinetics and 5'R3'P complex stability. C. R4·S44 is a ribozyme-substrate complex with four base pairs in H1 that was used to measure effects of modifications on intermolecular cleavage kinetics for G+1A and G+1Pu variants that retained too little ligation activity to support ligation-chase assays. D. Scheme for preparation of ribozyme variants with modifications at position 36 and 38 through ligation of three oligonucleotides.

FIGURE 3. Reaction scheme for measuring rate and equilibrium constants. The functional contributions of specific features of active site architecture were evaluated from the effects of nucleotide modifications on equilibrium and kinetic parameters measured in self-cleavage and ligation reactions. Changes in cleavage and ligation rates constants, $k_{\text{cleav}}$ and $k_{\text{lig}}$, reflect the effects of specific functional group modifications to the magnitude of the activation barrier to catalysis. G+1 is the essential residue at the 5' end of the 3' product RNA that mediates interdomain tertiary interactions (8). Tertiary structure stability is a primary determinant of the internal equilibrium, $K_{\text{eq}}^{\text{int}}$, between cleavage and ligation of bound products (13,14). Equilibrium dissociation constants, $K_d^{5'R\cdot3'P}$, for the complex that forms between 5' ribozyme and 3' product RNAs probe the contributions of functional groups to the stability of product binding in the ground state. Assays used to measure kinetic and equilibrium parameters are described in Experimental procedures.
TABLE 1. Synthetic oligoribonucleotides

| Sequence             | Modified nucleotides         | Notes                                  |
|----------------------|-----------------------------|----------------------------------------|
| AB(3'GA3)G4A         | 5'CAAGAUGAGAAGCCACCAGAGAAACACAAAGCGAA3' |                            |
| LR43AAΔB(5'GA3)C+6U  | 5'CGAAAGGUGCUGGUGGGAACACCCUGGCAGUCCAUAGAUC3' |                            |
| BdA38                | 5'AGCUUGGUDUAUACCUGGUACGAC3' |                                        |
| BA38dX               | 5'AGCUUGGUXUAUUACCUGGUACGAC3' (X = abasic deoxynucleotide) |                            |
| BA38Pu               | 5'AGCUUGGUPuUAUACCUGGUACGAC3' (Pu = purine) |                            |
| BG36I                | 5'AGCUUGIUAUUAUUACCUGGUACGAC3' (I = inosine) |                            |
| S43                  | 5'CCCACCUGGCAGUCCAC3' |                                        |
| S44G+1Pu             | 5'CCCACCUGGCAPuUCCACC3' (Pu = purine) |                            |
| S44G+1A              | 5'CCCACCUGGCAUCCACC3' |                                        |
| 3'P6C+6U             | 5'GUCCAUCU3' |                                        |
| 3'P6C+6U, dG+1       | 5'dGUCCAUCU3' |                                        |
| 3'P6C+6U, mG+1       | 5'mGUCCAUCU3' (mG = 2'-OMe guanosine) |                            |
| 3'P6C+6U, G+I        | 5'IUCCAUCU3' (I = inosine) |                                        |
| 3'P6C+6U, G+1(2AP)   | 5'(2AP)UCCAUCU3' (2AP = 2-aminopurine) |                            |
| 3'P6C+6U, G+1Pu      | 5'PuUCCAUCU3' (Pu = purine) |                                        |
| 3'P6C+6U, G+1A       | 5'AUCCAUCU3' |                                        |
| 3'P6C+6U, ΔG+1       | 5'UCCAUCU3' |                                        |
| 3'P7C+6U(3'G), G+1Pu | 5'PuUCCAUCUUGG3' (Pu = purine) |                            |
| 3'P7C+6U, G+1A       | 5'AUCCAUCUUGG3' |                                        |
| 3'P7C+6U, ΔG+1       | 5'UCCAUCUUGG3' |                                        |
| 3'P3C+6U             | 5'GUCCAUGAACC3' |                                        |
| 3'P3C+6U, dG+1       | 5'dGUCCAUGAG3' |                                        |
| 3'P3C+6U, mG+1       | 5'mGUCCAUGAG3' (mG = 2'-OMe guanosine) |                            |
| 3'P3C+6U, G+1A       | 5'AUCCAUGAG3' |                                        |
| 3'P3C+6U, G+1(2AP)   | 5'(2AP)UCCAUG3' (2AP = 2-aminopurine) |                            |
| 3'P3C+6U, G+1I       | 5'IUCCAUG3' (I = inosine) |                                        |

Nucleotide modifications that alter active site functional groups are indicated in bold font. Sequence changes that facilitate measurement of kinetics and equilibrium parameters are shown in regular font.
TABLE 2. Effects of modifications of G+1 interactions on complex stability and catalysis

| Complex  | $k_{\text{cleavage}}$ | $k_{\text{ligation}}$ | $K_{\text{eq int}}$ | $K_d$ | $\Delta G^{5'R-3'P}$ | $\Delta G^{5'R-3'P}_{15^\circ C}$ | $\Delta C$ |
|----------|-----------------------|-----------------------|---------------------|-------|----------------------|-------------------------------|---------|
| 5'R-3'P6 | 0.24±0.01             | 8.5±0.4               | 36±4$^d$            | 6.3±1.5 | -10.8±0.2           | -10.9±0.2                      | 20.0±0.1 |
| R4-S43   | 0.27±0.02             |                      |                    |       |                      |                               |         |
| 5'R-3'P6G+1Pu | (4.8±1.4)$\cdot$10$^{-4}$ | 0.29±0.01$^c$ | 590±130$^j$ | -8.2±0.2 | +2.6               |                                 |         |
| 5'R-3'P7G+1Pu | (5.4±0.4)$\cdot$10$^{-4}$ | 0.39±0.02$^c$ | 20±3$^j$   | -10.2±0.1 | +0.65             |                                 |         |
| R4-S44G+1Pu | (2.7±0.2)$\cdot$10$^{-4}$ |                      |                    |       |                      | 23.9±0.1                       | +3      |
| 5'R-3'P7G+1A | (1.4±0.2)$\cdot$10$^{-4}$ | 0.43±0.04$^c$ | 23±4$^j$   | -10.1±0.1 | +2.75             | 24.3±0.1                       | +4      |
| R4-S44G+1A | (9.3±0.4)$\cdot$10$^{-5}$ |                      |                    |       |                      | 24.5±0.2                       | +4      |
| 5'R-3'P7ΔG+1 |                      |                      | 20±4$^j$   | -10.2±0.1 | +2.65             |                                 |         |
| 5'RC25U-3'P6G+1Pu | 0.045±0.006 | 0.075±0.01 | 1.7±0.2$^d$ | 95±9$^k$ | -9.3±0.1           | +1.5                           | 21.0±0.1 |
| 5'R-3'P6G+1(2AP) | 0.017±0.001 | 0.033±0.003 | 2.0±0.4$^d$ | 320±60$^b$ | -8.6±0.2           | +2.2                           | 21.5±0.1 |
| 5'RC25U-3'P6 | 0.016±0.001 | 0.019±0.002$^m$ | 1.2±0.1$^c$ | 210±30$^k$ | -8.8±0.1           | +2.0                           | 21.5±0.1 |
| 5'R-3'P6G+1I | 0.037±0.004 | 0.17±0.03  | 4.6±1.1$^d$ | 460±120$^k$ | -8.4±0.2           | +2.45                          | 21.1±0.1 |
| 5'RC25U-3'P6G+1A | 0.042±0.001 | 0.43±0.02 | 10±1$^d$   | 50±6$^c$ | -9.6±0.1           | +1.25                          | 21.0±0.1 |
| 5'RC25U-3'P6G+1(2AP) | 0.21±0.01 | 3.8±0.2   | 18±1$^d$   | 19±2$^k$ | -10.2±0.1          | +0.65                          | 20.1±0.1 |

$^a$3'P6 or 3'P7 RNAs were used for $k_{\text{ligation}}$ and $K_d$ measurements.
$^b$Measured using ligation chase experiments with 5'R and 3'P3 RNAs.
$^c$k_{\text{ligation}}=k_{\text{obs ligation}}/k_{\text{cleavage}}$ with $k_{\text{obs ligation}}$ measured at saturating RNA concentrations and $k_{\text{cleavage}}$ values
obtained from ligation chase experiments.
$^d$K_{\text{eq int}}=k_{\text{ligation}}/k_{\text{cleavage}}$.
$^e$K_{\text{d}}^{5'R-3'P}=FLR_{\text{max}}/(1-FLR_{\text{max}})$, with FLR_{\text{max}} calculated using Equation 1 by assuming that $F_{\text{active}}=0.6$.
$^f$K_{\text{d}}^{5'R-3'P}=K_{\text{d,app}}^{5'R-3'P}$ in Equation 2.
$^g$ΔG^{5'R-3'P}_{15^\circ C}=-RT \ln (1/K_{\text{d}}^{5'R-3'P})$, where $R$ is the gas constant and $T$ is the temperature in degrees Kelvin.
$^h$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, unmodified
$^i$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, modified
$^j$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, unmodified
$^k$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, modified
$^l$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, unmodified
$^m$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, modified
$^n$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, unmodified
$^o$ΔG^{5'R-3'P}_{15^\circ C}=ΔG^{5'R-3'P}_{6}$, modified
$^p$Determined from the RNA concentration dependence of observed ligation rates.
$^q$Determined using gel mobility shift assays.
$^r$Calculated from $K_{\text{eq int}} \times k_{\text{cleavage}}$. 

Functional Analysis of Hairpin Ribozyme Architecture
TABLE 3. Contributions of nucleobase-ribose interactions and stacking to complex stability and catalysis

| Complex          | $k_{\text{cleavage}}$  | $k_{\text{ligation}}$ | $K_{\text{eq}}^\text{int}$ | $K_d$ | $\Delta G^{5'R·3'P}_{\text{15'C}}$ | $\Delta \Delta G^{5'R·3'P}_{\text{15'C}}$ |
|------------------|-------------------------|------------------------|-------------------------------|-------|-----------------------------|----------------------------------|
|                  | (min$^{-1}$)             | (min$^{-1}$)            |                               | nM    | kcal/mol                     | kcal/mol                          |
| 5'R·3'P6dG+1     | 0.34±0.03               | 3.8±0.2                | 11±1                          | 12±3$^k$ | -10.4±0.2                   | +0.45                            |
| 5'RG361·3'P6     | 0.32±0.04               | 4.3±0.6                | 14±2                          | 20±5$^i$ | -10.2±0.2                   | +0.64                            |
| 5'R·3'P6mG+1     | 0.12±0.01               | 1.2±0.2                | 10±2                          | 31±7$^l$ | -9.9±0.2                    | +0.95                            |
| 5'RdA38·3'P6     | 0.13±0.01$^l$           | 1.5±0.03$^l$           | 12±0.6$^l$                    | 9.2±2.5$^j$ | -10.6±0.2                   | +0.25                            |
| 5'RA38dX·3'P6    | 2.1·10$^{-5}$           | 2.5·10$^{-5}$          | 1.2$^j$                       | 88±37 | -9.3±0.3                    | +1.55                            |

$^a$3'P6 or 3'P7 RNAs were used for $k_{\text{ligation}}$ and $K_d$ measurements.
$^b$Measured using ligation chase experiments with 5'R and 3'P3 RNAs.
$^c$k_{\text{ligation}}=k_{\text{obs, ligation}}/k_{\text{cleavage}}$ with $k_{\text{obs, ligation}}$ measured at saturating RNA concentrations and $k_{\text{cleavage}}$ values obtained from ligation chase experiments.
$^d$K_{\text{eq}}^\text{int}=k_{\text{ligation}}/k_{\text{cleavage}}$
$^e$K_d$^{5'R·3'P}$=K_{d,app}$^{5'R·3'P}$x(K_{\text{eq}}^\text{int}$+1)^$
$^f$ΔG$^{5'R·3'P}_{\text{15'C}}$=−RT ln (1/K_d$^{5'R·3'P}$), where R is the gas constant and T is the temperature in degrees Kelvin.
$^g$ΔG_{15'C}=ΔG_{5'R·3'P, unmodified}^{5'R·3'P, modified}$ or $ΔG_{5'R·3'P, modified}^{5'R·3'P, unmodified}$ - ΔG_{5'R·3'P, modified}^{5'R·3'P, unmodified} 2 kcal/mol for complexes with 3'P7 instead of 3'P6, as explained in the text.
$^i$ΔG_{15'C}=−RT ln (k_{\text{cleavage}}/k_B T)$ where $k_B$ is Boltzmann’s constant, and $k_{\text{cleavage}}$ is the self-cleavage rate constant at $T$=288°C.
$^j$ΔG_{15'C}=ΔG_{15'C}^{15'C}$ or ΔG_{15'C}^{15'C} - ΔG_{15'C}^{15'C}$modified$.
$^k$Determined from the RNA concentration dependence of observed ligation rates.
$^l$Determined using gel mobility shift assays.
$^m$Value taken from Kuzmin et al., 2005.
FIGURE 2

A

LR43

B

5' R4 3' P6

C

R4 S44

D

B + AB(3'GA3)G4A + LR43ΔAΔB(5'GA3)C+6U

LR43(5'ΔG)G4A, C+6U
FIGURE 3

$5' R + 3' P \rightleftharpoons \frac{K_d^{5'R \cdot 3'P}}{k_{on}} \frac{1}{k_{off}}$

$5' R \cdot 3' P \rightleftharpoons K_{eq^{int}} \frac{1}{k_{lig}} \frac{1}{k_{cleav}}$

$5' R \cdot 3' P \rightleftharpoons LR$
Functional analysis of hairpin ribozyme active site architecture
Joseph W. Cottrell, Yaroslav I. Kuzmin and Martha J. Fedor

J. Biol. Chem. published online March 9, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M700451200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts