Reconstitution of Hairpin Ribozyme Activity following Separation of Functional Domains*

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The hairpin ribozyme is a 50-nucleotide RNA enzyme of unknown three-dimensional structure. Here, we demonstrate that interdomain interactions are required for catalytic function by reconstitution of activity following separation of an essential, independently folding domain (loop B) from the substrate binding strand at a helical junction. The resulting construct relies on long range tertiary contacts for catalysis. For this work, we used an optimized ribozyme and substrate, which included sequence changes to minimize the formation of nonproductive conformational isomers. Kinetic analysis was carried out using both single and multiple turnover methods and shows that the catalytic efficiency (kcat/Km) of the reconstituted ribozyme is 104-fold lower than that of the intact ribozyme. The decrease in kcat/Km results entirely from a 104-fold increase in the apparent Km, whereas the kcat parameter is essentially unchanged. Therefore, cleavage chemistry appears to be unimpaired, but the reaction is limited by the productive assembly of the two domains. Our results strongly support a previously proposed model in which the catalytic topology of the ribozyme contains a bend at a helical junction.

The hairpin ribozyme is derived from the satellite RNA of the tobacco ringspot virus and catalyzes the reversible, site-specific cleavage of RNA substrates, generating 5'-OH and 2',3'-cyclic phosphate termini (1-3). Nucleotides and functional groups that are essential for catalytic function have been identified by several methods, including mutagenesis (4, 5), in vitro selection (6-9), and the introduction of modified nucleotides (10-12). Interestingly, essential elements of the ribozyme are concentrated within a large asymmetrical internal loop (loop B) and a smaller symmetrical loop formed through binding of the substrate to the substrate binding strand (SBS)† (see Fig. 1). The finding that many of the essential determinants reside within loop B strongly suggests that this portion of the molecule interacts with the rest of the ribozyme-substrate complex during catalysis. Linker insertion (13, 14) and chemical modification studies (15) of the helix 2-3 junction indicate that helix 2 is not coaxially stacked upon helix 3, i.e. the ribozyme-substrate complex has a bend between helices 2 and 3. Such a bend would presumably be required to bring essential elements in loop B proximal to the cleavage site.

Local interdomain interactions within loop B have been studied by UV cross-linking (16) and chemical modification analysis (15). These studies indicate that a photosensitive structural motif occupies the segment of loop B that is proximal to helix 3. This portion of the ribozyme is strikingly similar to the conserved central domain of viroids (17), to loop E of eukaryotic 5'S RNA, and to the sarcin-ricin loop of 26 S RNA. The latter two structures have been analyzed using NMR methods (18-20). These structures are comprised of non-canonical G-A, A-U, and A-A base pairs across the loop and result in a helical conformation with accessible major and minor grooves. The reason a common structural motif is found in several functionally distinct RNAs is not yet clear, but it has been proposed that the structure may serve as a common “docking module” for RNA-RNA or RNA-protein interactions (20, 21). In the hairpin ribozyme, the cross-linkable loop B structure is clearly essential for catalytic function. Furthermore, results of chemical modification experiments suggest that magnesium-dependent higher order structure is superimposed on the photoactive motif (15).

Cross-linking and chemical modification results indicate that the loop B domain of the hairpin ribozyme, defined as helix 3, loop B, and helix 4, folds into its correct structure independently of substrate and SBS (15, 16). We took advantage of this property to search for functional interactions between loop B and a duplex consisting of substrate bound to the SBS (S-SBS). Using this strategy, we have been able to reconstitute the cleavage reaction. The interdomain interactions demonstrated by this reconstitution are likely to be tertiary contacts, since little potential for formation of canonical base pairs exists between the domains and no phylogenetic evidence for interdomain secondary structure could be obtained by analyzing a large number of sequence variants (6, 7, 9).

EXPERIMENTAL PROCEDURES

RNA Synthesis—RNA molecules comprising the loop B domain were transcribed with T7 RNA polymerase in vitro as described (16, 22). Substrate and SBS RNAs and DNA oligonucleotides were synthesized using standard solid-phase phosphoramidite chemistry, deprotected as described (23), and purified by polyacrylamide gel electrophoresis.

Single Turnover Kinetics—Single turnover kinetics were performed essentially as described (24). The optimal magnesium ion concentration for the reaction was determined by measuring initial reaction velocities as a function of MgCl2 concentration and was found to be 50 mM or greater, which is approximately 4-fold higher than that of the intact ribozyme (2, 25). An increase in the observed optimal magnesium ion concentration is common for reactions that rely on tertiary interactions (26, 27). All kinetic analyses were performed in magnesium excess (100 mM MgCl2). Each experiment was performed at least twice, and variences of ±10% were obtained in duplicate experiments. Single turnover reactions were performed with 10 nM 5'-32P-end-labeled substrate, which was prebound to excess SBS by equilibrating at 37 °C for 5 min in 50 mM Tris-HCl (pH 7.5), 100 mM MgCl2. These conditions were shown by nondenaturing gel electrophoresis to result in essentially quantitative association of substrate with SBS. Reactions were then initiated by the addition of the loop B domain, also pre-equilibrated in the same buffer. Reactions were quenched by the addition of 4 volumes of formamide, which was shown to completely arrest the reaction. Samples were then...
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Results were quantitated with a GS-250 radioimaging system
(Bio-Rad) and plotted as a function of the remaining fraction
of substrate present versus time, yielding slopes equivalent
to the negative rate of the reaction (kcat). Initial velocities
were calculated over a time span that was within the linear
range (≤30% cleavage) and were plotted as a function
of loop B concentration. Ks and kcat values were determined
by curve fitting to the Michaelis-Menten equation using
non-linear least squares regression analysis (kcat software,
Biometallics).

Multiple Turnover Kinetics—Multiple turnover kinetics were
performed under conditions identical to the single turnover
reactions except that the loop B domain was present at low
concentrations (100 nM) and loop A duplex (S-SBS) was in excess.

A variety of S-SBS concentrations (1–370 μM) was used, in
which a trace amount of the substrate (1 nM) was 5’-end-labeled.
When substrate concentrations were below 15 μM, SBS was
kept in excess (15 μM) to maintain substrate in the form
of bound duplex. For concentrations above 15 μM, SBS and substrate
were present at equimolar concentrations. Reactions were
incubated at 37 °C for up to 14 h. For the intact hairpin ribozyme,
much lower concentrations of substrate (0.01–1 μM) and ribozyme
(1–5 nM) were required, and reactions were incubated for 4 min.
All reactions were performed at 37 °C in the buffer described above.
Initial velocities were calculated for each substrate concentration,
and Ks and kcat values were determined as described above.

RESULTS AND DISCUSSION

The naturally occurring substrate sequence for the hairpin
ribozyme (UGACA ↓ GUCCUGUUU) is conformationally het-
erogeneous and migrates as multiple conformations on native
polyacrylamide gels (28). In general, it was found that confor-
formationally heterogeneous substrates and ribozymes showed
unsatisfactory kinetic behavior. To design a well behaved
substrate and SBS, we varied sequences in such a manner that the
conformational heterogeneity is minimized while maintaining
sequences essential for catalytic function (Fig. 1). Confor-
national homogeneity of the new substrate and SBS over a con-
centration range of 1 nM to 40 μM was confirmed by native gel
electrophoresis (data not shown). We utilized a stabilized loop
B domain in which helix 4 was extended by the addition of
a rate-enhancing U39C mutation (7, 9). These mod-
ifications each approximately double the catalytic efficiencies
of intact hairpin ribozymes and produce a loop B domain that
gives only one detectable conformation, which is the active and
cross-linkable structure (16, 30).

To reconstitute a cleavage reaction between separated loop
A and B domains, loop A duplexes were preformed by annealing
substrate to its cognate SBS. Cleavage activity was observed
only when the loop B domain was incubated with the S-SBS
duplex (Fig. 1B). No reaction could be detected in the absence
of the loop B domain or in the absence of the substrate binding
sequence, even upon extended incubation times. The reaction
occurs at the normal site, as evidenced by product mobility,
and no inaccuracy of cleavage is detectable. These results
demonstrate a functional interaction between the two domains
of the ribozyme-substrate complex.

The background rate of hydrolysis of substrate RNA was
measured in the absence of ribozyme, under buffer and tem-
perature conditions identical to those used in the Fig. 1 recon-
stitution experiment (data not shown). The measured rate was
approximately 10−6 min−1, which is similar to previously re-
ported values for RNA hydrolysis (31). Similar background
rates were observed in the presence of a 100-1000-fold excess
of SBS, indicating that formation of the loop A domain does not
result in an increased lability of the scissile bond. In these
latter experiments, 1 nM 5’-end-labeled substrate was
incubated with 100 and 1000 nM SBS for up to 48 h. Similarly, no
increased lability of the scissile bond was observed with
substrate incubated in the presence of loop B (5 μM) with no SBS
present.

Kinetic analysis of the bisected ribozyme construct provides
useful insights into interactions between the domains. To en-
sure that substrates remained bound to substrate binding
strands in the form of S-SBS duplexes, we first determined the
apparent Ks for the SBS in the ternary complex (Fig. 2). The
apparent Ks was observed to be approximately 70 nM for the
SBS, and saturation was observed in all cases where SBS
concentration was ≥5 μM. In these experiments, Ks was
observed to be independent of loop B concentration. To main-
tain the S-SBS duplex in subsequent experiments, we chose to
use an excess of SBS relative to S, such that SBS concentrations
were always 15 μM or greater.

We used both pre-steady state (single turnover) and steady
state (multiple turnover) kinetic analysis to describe the re-
constituted reaction. Single turnover reactions were performed
by using a trace amount of radioactive substrate prebound to SBS
and increasing amounts of the loop B domain. Loop B domain
was always in significant excess over substrate. The reaction
could not be fully saturated even when the concentration of
loop B was increased to 400 μM (Fig. 3, A–C). The observed
reaction velocities at 400 μM loop B are within 2–3-fold of the

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maximum velocity of the corresponding version of the intact ribozyme. First order rate constants obtained at eight different concentrations of loop B were plotted, and the resulting curve was fitted by non-linear least squares regression analysis to the Michaelis-Menten equation, yielding a $k_{cat}$ of 0.53 min$^{-1}$ and an observed $K_m$ of 270 μM (Fig. 3C and Table I). The value of $k_{cat}$ is essentially unchanged relative to that of the intact ribozyme, while $K_m$ for the reconstituted reaction is increased by a factor of 10$^4$. These results indicate that the reconstituted reaction is likely to be limited only by the productive association between the two ribozyme loop domains.

Multiple turnover kinetics were performed by incubating a small amount of the loop B domain in the presence of a large excess of the S-SBS duplex and monitoring initial reaction velocities. As in the pre-steady state analysis, we observe that the reaction requires very high concentrations of substrate to achieve maximum velocity. We find that the loop B domain is capable of cleaving multiple S-SBS duplexes, with a turnover rate ($k_{cat}$) of 0.13 min$^{-1}$ (Fig. 3D and Table I). This value is about 4-fold lower than the $k_{cat}$ value measured by single turnover kinetics. Therefore, it appears that reactions in the presence of a large excess (200–300 μM) of S-SBS are slightly less efficient than reactions with a corresponding excess of the loop B domain. This difference may be due to inhibition by high concentrations of S-SBS (for example, RNA aggregation or inhibition by excess substrate or SBS) or to a change in the rate-limiting step. The steady state analysis of the reconstituted reaction indicates that the loop B domain can cleave multiple S-SBS complexes, but reaction efficiency is limited by a 10$^4$-fold increase in $K_m$.

Reconstitution of activity based on tertiary interactions has been shown for large group I and group II ribozymes (26, 27, 32–35). Feldstein and Bruening (13) showed that a trans-ribozyme restored self-ligation activity to a conformationally constrained construct that had little or no intramolecular activity. This work showed that intermolecular interactions could enhance hairpin ribozyme activity but did not define the mode of interaction between the two ribozymes. The interdomain tertiary interactions in the hairpin ribozyme are surprisingly weak in comparison with those observed in the larger ribozymes. For example, domain V of the self-splicing a57 group II intron binds to the intron through tertiary interactions and has a $K_m$ of 300 μM (26). In contrast, our observed value of $K_m$ for the hairpin ribozyme domains (60–270 μM) correlates more closely with that of the interaction between guanosine substrate and the Tetrahymena group I intron, where a $K_m$ of 320 μM is observed (36, 37). Although we have not demonstrated that our $K_m$ corresponds to a dissociation constant, the notion of weak interdomain interactions is supported by direct analysis of complex formation on nondenaturing gels.3 In these experiments, formation of the S-SBS loop B ternary complex could not be detected, while S-SBS and S-ribozyme complexes were readily identified. Therefore, it appears that the interdomain tertiary interactions in the hairpin ribozyme may be limited to a small number of contacts. Additionally, it is likely that an entropic penalty accompanies domain separation, giving rise to high apparent $K_m$ values.

In light of the apparently high $K_m$ value for interdomain interactions in the hairpin ribozyme, the covalent linkage between the domains in the intact ribozyme may serve to increase greatly the local effective concentrations of the domains. Additionally, it is possible that the phosphodiester linkage partly constrains the orientation of the two domains, such that the frequency of productive interactions may be increased. It is interesting to note that the interdomain interactions described here require a strong bend at a helical junction in the intact ribozyme; this is unusual because coaxial stacking is a common feature of RNA structure and is energetically favorable (38). We expect that this reconstituted reaction will be useful in the identification of specific interdomain interactions and for analysis of the catalytic mechanism in the hairpin ribozyme system.

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