Metal Ion Dependence of Active-Site Structure of the *Tetrahymena* Ribozyme Revealed by Site-Specific Photo-cross-linking

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Abstract: The L-21 *Sca*I ribozyme derived from the *Tetrahymena* group I intron recognizes its RNA substrate by base pairing, forming the P1 duplex, and cleaves it in a Mg^{2+}-dependent transesterification reaction. In the presence of a substrate analog, photo-cross-linking of 5′-(azidophenacyl)-substituted L-21 *Sca*I ribozyme gives only two cross-linked products over a wide range of Mg^{2+} concentrations. Product **1** corresponds to the catalytically active RNA structure at high Mg^{2+} concentration, and product **3** to an inactive structure formed in the absence of Mg^{2+}. The observed change in the ratio of cross-linked species **1** versus **3** as a function of Mg^{2+} concentration shows high cooperativity in the folding of the P1 duplex with the catalytic core of the ribozyme (Hill constant = 8 ± 2). In addition, the site-specific photo-cross-linking detects for the first time a difference between the Mg^{2+}-folded and the Ca^{2+}-folded ribozyme structures, a difference which involves the positioning of P1. Introduction of cross-link **1** into the RNA does not render it catalytically active in the presence of Ca^{2+}, consistent with Mg^{2+} being important in the chemical step in addition to its contribution to the correct positioning of P1.

Introduction

RNA enzymes (ribozymes) require specific metal ions for their catalytic activities.[1] For example, the L-21 *Sca*I RNA derived from the *Tetrahymena* group I intron requires Mg^{2+} or Mn^{2+} both for folding into its active structure and for the chemical step of catalysis.[2] The folding requirement has been explored using free radicals generated by EDTA–Fe(II) as a cleavage reagent.[2a,3] Because the catalytic core resides in the interior of the ribozyme structure and has reduced accessibility to this solvent-based cleavage reagent, EDTA–Fe(II) is not a sensitive probe of structure within the core. Here we use photo-cross-linking as an active-site structural probe to monitor the metal ion dependence of the tertiary folding around the internal guide sequence (IGS) of the *Tetrahymena* ribozyme.

An azidophenacyl moiety was tethered to the 5′-phosphate of the phylogenetically conserved G22 of the L-21 *Sca*I (*Tetrahymena*) ribozyme (Figures 1 and 2). G22 is the nucleotide at the 5′-end of the IGS, the sequence which base pairs with the RNA strand that is cleaved in the P1 duplex.[4] Photo-cross-linking of the ribozyme under conditions appropriate for its catalytic activity (with the P1 duplex formed) showed G22 to be proximal to the conserved adenosines between P4 and P5 (A114/A115, cross-link **1**).[5] In the absence of an RNA substrate or substrate analog, G22 cross-linked to the A88 (**2a**) and U300 (**2b**) sites instead. The absence of Mg^{2+} caused the 5′-end of the IGS to cross-link to the C102 (**3**) site (U101/C102/A103, Figure 2). By monitoring the changes in cross-link site upon changing the concentration of Mg^{2+} and upon substituting Mg^{2+} with other divalent metal ions, we have now gained new information about how metal ions participate in RNA folding, especially in the docking of the P1 helix to the catalytic core of the ribozyme.

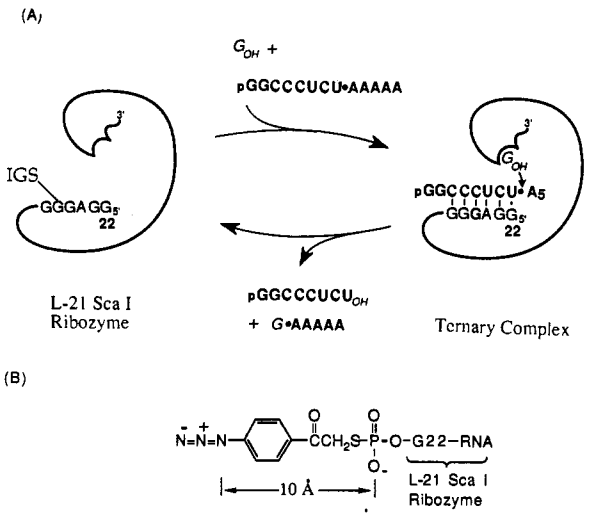

Figure 1. (A) Endonuclease reaction catalyzed by the ribozyme. The RNA substrate (GGCCCUCUAAAAA) base pairs with the internal guide sequence (IGS: GGAGGG) of the ribozyme. After ribozyme-catalyzed cleavage, the same base pairs persist before oligonucleotide GGCCCUCU (cleavage product **P**) dissociates from the IGS. Filled circles in oligonucleotides represent phosphodiester bonds cleaved or formed during the reaction. (B) Azidophenacyl group coupled to the 5′-end of the L-21 *Sca*I (*Tetrahymena*) ribozyme through a thiophosphate. Reproduced by permission from ref 5 (© AAAS).

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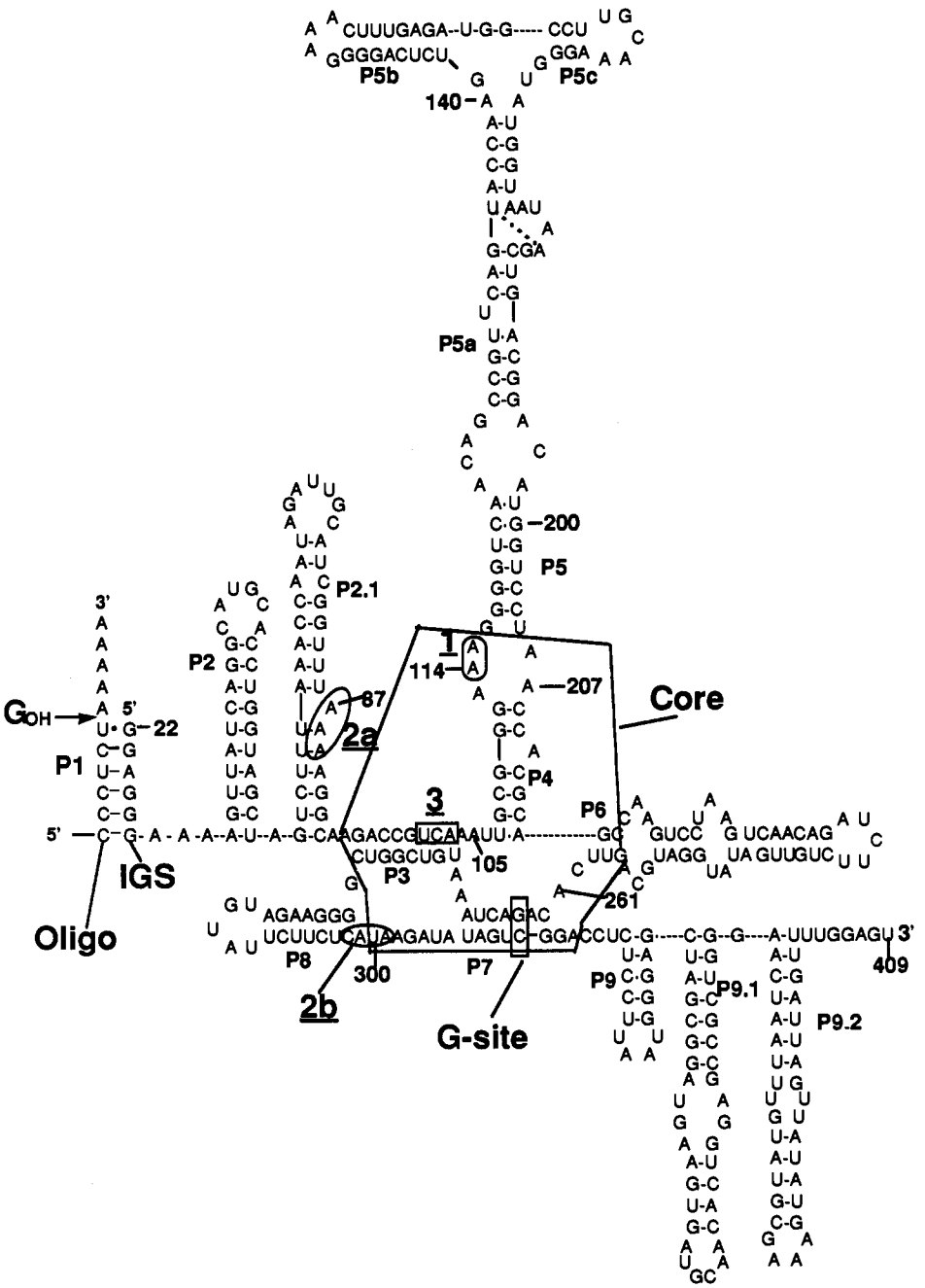

Figure 2. Sites of cross-links by the nitrene group at the 5′-end (G22) of L-21 *Sca*I ribozyme,[5] superimposed onto the secondary structure diagram of the RNA.[14] (P1–P9) Paired (duplex) regions common to group I introns. In this multiple-turnover version of the ribozyme, the P1 helix is formed by base pairing of an RNA oligonucleotide (CCCUCUAAAAA in this figure) to the IGS. Because tertiary interactions are superimposed on base pairing, complementary RNA oligonucleotides bind very tightly to the IGS of the ribozyme ($K_d \simeq 0.3$ nM at 42 °C):[15] (**1**) nucleotides cross-linked in the presence of **P** (GGCCCUCU); (**2a** and **2b**) cross-links in the absence of **P**; (**3**) cross-links in the absence of Mg^{2+}.

Results and Discussion

The 5′-(azidophenacyl) L-21 *Sca*I ribozyme was irradiated in the presence of a saturating concentration (3 μM) of the oligonucleotide GGCCCUCU, which base pairs to the IGS to form the P1 duplex (Figures 1A and 2). As the concentration of Mg^{2+} was increased from 0 to 2.5 mM, the cross-link between the derivatized G22 and the C102 site was eliminated while the cross-link to A114/A115 appeared (Figure 3A). A minor cross-linked product (*) (~6% of **3**) formed concomitantly with cross-linked product **3**. The location of this minor cross-link was not identified, but it was presumably adjacent to the C102 site because both cross-links resulted in similar mobility on a denaturing gel.[5] No other cross-links occurred to any significant extent over the entire Mg^{2+} concentration range. The same cross-linked species and the same transition midpoint ($[Mg^{2+}]_{1/2} = 1.6$ mM) were also observed when the Mg^{2+} concentration was decreased by dilution (Figure 3B). Therefore, the two conformers leading to the cross-links at the C102 site and A114/A115 are in thermodynamic equilibrium.

The catalytic activity of the ribozyme was also assessed by measuring k_{cat}/K_m for cleavage of an oligonucleotide substrate as a function of Mg^{2+} concentration (see Experimental Section). The ribozyme structure required for cross-link **1** corresponds to the catalytically active structure, because the yield of cross-link **1** and the kinetic parameter k_{cat}/K_m have the same dependence on Mg^{2+} concentration (Figure 3B). Cross-link **3**, on the other hand, corresponds to a catalytically inactive structure.

The observation of only two major cross-link sites over a wide range of Mg^{2+} concentration (Figure 3) suggests that the folding of the L-21 *Sca*I ribozyme with Mg^{2+} is a highly cooperative

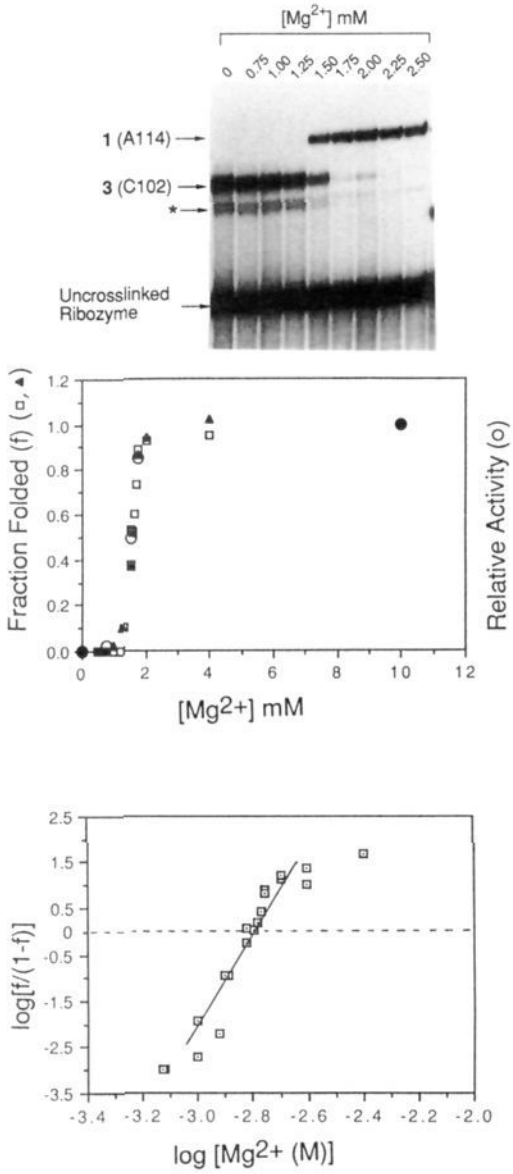

Figure 3. Effect of Mg^{2+} concentration on the photo-cross-linking of ^{32}P-labeled 5′-azidophenacyl L-21 *Sca*I ribozyme. (A, top) Autoradiogram of a 6% polyacrylamide–7 M urea gel: *, a minor cross-linked product that coappeared with **3** (see text). (B) Relative extent to which the L-21 *Sca*I ribozyme achieved the final folded state around G22 as a function of Mg^{2+} concentration. A value of fraction folded refers to the fraction of cross-link **3** converted to cross-link **1** (cross-link **1** correlates with the molecules that have achieved the final folded state): open squares, data obtained with increasing Mg^{2+} concentration; closed triangles, data with Mg^{2+} concentration decreased by dilution; open circles, the relative endonuclease activity of the ribozyme $[(k_{cat}/K_m)_{rel} = (k_{cat}/K_m)_{Mg}/(k_{cat}/K_m)_o$, where $(k_{cat}/K_m)_o = 3.0 \times 10^7\ M^{-1}\ min^{-1}$, the endonuclease activity at 10 mM Mg^{2+}}; closed circles, fraction folded and ribozyme activity normalized to unity at 10 mM Mg^{2+}. (C, bottom) Hill analysis of the folding data represented in part B. The line describes the relation $\log[f/(1-f)] = h \log [Mg^{2+}] - \log K_d$, where h (Hill constant) is the slope of the line at midtransition, f is the fraction folded, and K_d is the dissociation constant for the RNA·$(Mg^{2+})_h$ complex. It is assumed that $[Mg^{2+}]_{free} = [Mg^{2+}]_{total}$.

process which can be approximated by a two-state model: the cross-link at the C102 site corresponds to the "unfolded" state, and the cross-link at A114/A115 corresponds to the "folded" state. The "unfolded" and "folded" states differ at the level of tertiary folding. By "unfolded" we mean that the tertiary structure around the 5′-end of the IGS is not formed, but base-pairing interactions should occur under our experimental conditions because of the presence of high concentration of salts (100 mM NaCl and up to 0.75 mM Mg^{2+}). The Mg^{2+} dependence of the cross-linking remained similar even when the NaCl concentration was increased to 500 mM, as expected if the change in the cross-link sites as a function of Mg^{2+} reflects the degree of tertiary folding and not the formation of the secondary structure. Because the cross-linking agent was present on the IGS, our conclusions about folding refer to the position of the IGS relative to the remainder of the RNA.

To assess the cooperativity of this folding transition, we performed Hill analysis of three independent sets of data. The Hill constant was measured to be 9.3 (Figure 3C), 7.7, and 8.0, an average of 8 with a maximum uncertainty of 2. This result indicates that at least eight additional Mg^{2+} ions (beyond those already bound in the unfolded state) are required to promote the cooperative folding of the region of the ribozyme in proximity to the IGS.[6] A previous study of EDTA–Fe(II) cleavage of the L-21 *Sca*I ribozyme as a function of Mg^{2+} also led to the conclusion that the tertiary folding of the RNA is a cooperative process.[2a] In the EDTA–Fe(II) study, the Hill constant was derived from "fraction-folded" values that were averaged over various nucleotide positions along the ribozyme sequence. The folding around the IGS was not monitored due to difficulty in quantitation of cleavage near the end of the molecule.

The L-21 *Sca*I RNA is active as an endonuclease only in the presence of Mg^{2+} or Mn^{2+}. However, other group IIA divalent metal ions such as Ca^{2+} and Sr^{2+} have been found to permit tertiary folding of the ribozyme.[1c,2a] It remained possible that the tertiary structure folded with Mg^{2+} might differ from the structure folded with other group IIA divalent ions in some way that was not revealed by previous structural analysis using EDTA–Fe(II). When 10 mM Mg^{2+} was replaced by the same concentration of Ca^{2+}, the cross-link to A114/A115 decreased dramatically (92%) and a cross-link to the A88 site appeared (Figure 4). Because the cross-link at the A88 site is one of the two cross-links observed when the IGS is unoccupied, it seemed that the switch to Ca^{2+} might simply promote dissociation of the bound oligonucleotide. But the binding affinity of the oligonucleotide **P** (GGCCCUCU) to the ribozyme decreases only from $K_d \cong 1$ nM in Mg^{2+} to 40 nM in Ca^{2+}.[7] The concentration of the oligonucleotide **P** used in the experiment was 3 μM, much higher than the measured K_d, and even at 30 μM the same cross-linking pattern was observed (data not shown). Furthermore, cross-linked product **2b** (which accompanies **2a** in the absence of **P**) was not observed in the presence of Ca^{2+}. Taking all these facts together, we conclude that the IGS is saturated by oligonucleotide **P**. The cross-link at the A88 site has been suggested to represent the "open conformation" of the ribozyme bound with oligonucleotide substrate, an inactive conformation that is an alternative to the catalytic conformation represented by cross-link **1** ("closed conformation").[5,8] Thus, the observation of different cross-link patterns for the L-21 *Sca*I RNA with Mg^{2+} versus Ca^{2+} provides evidence for different positions of P1 relative to the catalytic core depending on which divalent cation is present.

The failure of P1 to be properly positioned in the presence of Ca^{2+} was readily reversible. Addition of 0.5 mM Mg^{2+} to the Ca^{2+}-containing solution restored efficient cross-linking of G22 to A114/A115 (Figure 5, lanes 5 and 6) and restored the endonuclease activity of the ribozyme (data not shown). The concentration of 0.5 mM Mg^{2+} by itself was too low to lead to any extent of tertiary folding of the RNA detectable by cross-links (Figure 5, lane 8). In addition, the cross-link sites at U300

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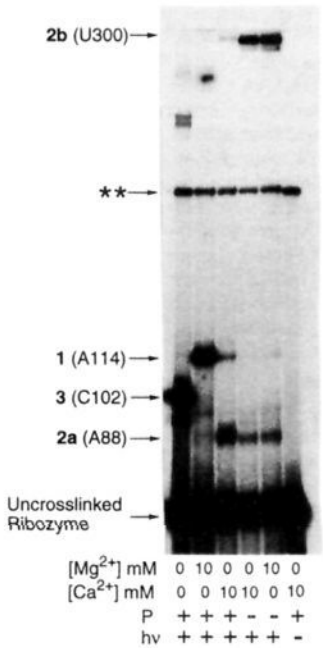

Figure 4. Photo-cross-linking of 5′-azidophenacyl L-21 *Sca*I ribozyme in the presence of Mg^{2+} versus Ca^{2+} ions: $h\nu$ (–), starting material incubated in the presence of 10 mM Ca^{2+} but without subsequent UV irradiation; The concentration of RNA GGCCCUCU (**P**) is 3 μM (+) or zero (–); **, an RNA species particular to this preparation; it is presumably an aggregated form of the un-cross-linked RNA because, after isolation, it comigrated with the un-cross-linked ribozyme on a 7 M urea gel and, when used as an RNA template, did not stop reverse transcriptase in primer extension.

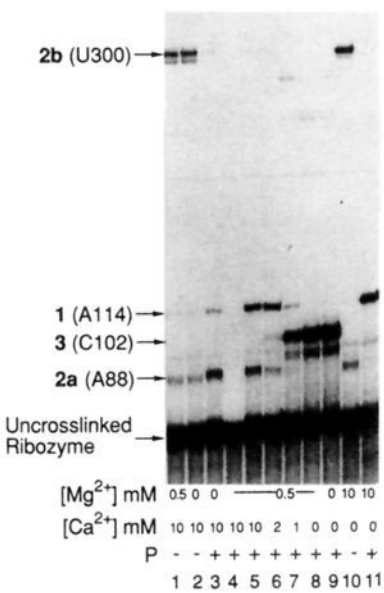

Figure 5. Photo-cross-linking of 5′-azidophenacyl L-21 *Sca*I ribozyme in a mixture of Mg^{2+} and Ca^{2+} ions. **P** is defined as in Figure 4: lane 4, no UV irradiation.

and at A88 observed in the absence of oligonucleotide **P** remained approximately unchanged when Mg^{2+} was substituted by Ca^{2+} (Figure 4). These results suggest that Mg^{2+} and Ca^{2+} promote formation of the same overall structure of the ribozyme, differing only in regard to positioning of the P1 duplex.

It was then of interest to test whether Ca^{2+} could substitute for Mg^{2+} in the enzymatic cleavage reactions if the L-21 *Sca*I RNA was first cross-linked to A114/A115 to artifically position P1 at the proper site. However, although cross-linked species **1** was catalytically active in the presence of Mg^{2+} [(k_{cat}/K_m) with **1** is about 1/2 that with the un-cross-linked L-21 *Sca*I RNA], it was inactive in the presence of 10 mM Ca^{2+} (data not shown). A reasonable explanation is that Mg^{2+} is required not only for the correct positioning of the P1 helix but also for participation in the chemical cleavage step. Using the cross-linked form meets only the first requirement and is not sufficient to confer activity on the ribozyme folded with Ca^{2+}. Alternatively, tethering the IGS in the correct vicinity with a cross-link may not position it in quite the active conformation in the absence of Mg^{2+}.

Cooperative folding of RNA with binding of Mg^{2+} (or Mn^{2+}) has been observed previously with transfer RNA.[9] Mg^{2+} binding is cooperative only with tRNA that is not in its native tertiary structure, which can be explained by several Mg^{2+} ions acting as "allosteric effectors" to convert the non-native molecule into its native conformation.[9b,10] This is exactly the interpretation we have for our cross-linking data: as Mg^{2+} is added, the non-native ribozyme (secondary structure only) folds cooperatively into its native, catalytically active conformation (secondary plus tertiary structure). One apparent difference is that the native tRNA structure can be formed in the presence of monovalent cations only,[9c,10c] whereas the native structure of the *Tetrahymena* ribozyme requires divalent cations.[1c,i,2a,3a]

In vitro selection has been employed by Lehman and Joyce to select mutants of the ribozyme that are catalytically active when folded with Ca^{2+}.[11] Their studies have demonstrated that mutations at only a few sites are sufficient to confer ribozyme activity in the presence of Ca^{2+}. It will be interesting to test whether the cross-linking pattern of these mutant ribozymes remains the same when Mg^{2+} is substituted by Ca^{2+}.

In summary, we have demonstrated that site-specific photo-cross-linking can be used as a structural probe to study the metal ion dependence of the tertiary folding of an RNA molecule. We have provided evidence that the positioning of the IGS relative to the catalytic center of the *Tetrahymena* ribozyme is a highly cooperative Mg^{2+}-promoted folding process which can be approximated by a two-state model. Other divalent metal ions such as Ca^{2+} may give the same overall structure but fail to properly position the P1 duplex or to allow the chemical step to proceed.

Experimental Section

Materials. Magnesium chloride hexahydrate (99.995%) and calcium chloride hydrate (>99.99%) were obtained from Aldrich. The enzymatic synthesis and purification of oligonucleotide substrate GGCCCUC-UAAAAA and 5′-exon analog GGCCCUCU were as described previously.[5]

Guanosine 5′-phosphorothioate (GMPS) was synthesized by reaction of guanosine (Sigma) with thiophosphoryl chloride (Aldrich) in triethyl phosphate (Aldrich).[12] After purification by HPLC, the GMPS was used as the initiating nucleotide for the *in vitro* transcription of L-21 *Sca*I RNA by bacteriophage T_7 RNA polymerase as described,[4a] except that 4 mM GMPS and 0.25 mM GTP were used. Most studies were performed with [^{32}P]RNA, prepared by inclusion of [α-^{32}P]ATP in the transcription reaction. The transcribed RNA was purified by electrophoresis on a 6% polyacrylamide–7 M urea gel, visualized by autoradiography, eluted from the gel, precipitated with ethanol, and resuspended in 40 mM sodium bicarbonate (pH 8.5). A small volume of methanol saturated with azidophenacyl bromide was then added so that the final concentration of azidophenacyl bromide was 5–50 mM. After 1 h at room temperature, excess azidophenacyl bromide was removed by extraction with two volumes of phenol and the 5′-azido L-21 *Sca*I RNA was recovered by ethanol precipitation.

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Photo-Cross-Linking. Uniformly ^{32}P-labeled 5′-azido L-21 *Sca*I RNA (30 nM) in 30 mM Tris (pH 7.5), 100 mM NaCl, Mg^{2+} or Ca^{2+} of specific concentration was preincubated at 42 °C for 15 min and then irradiated at 42 °C for 10 min (312 nm, Model FBTIV-816 transilluminator, Fisher Scientific) with light filtered through Pyrex glass. Samples were immersed in a temperature-controlled water bath during irradiation. The reaction solution was then mixed with an equal volume of buffer [10 M urea, 0.2 × TBE (1 × TBE: 100 mM Tris, 100 mM boric acid, 2 mM EDTA, pH 8.3, 0.02% each bromophenol blue and xylene cyanol, 40 mM EDTA] and subjected to gel electrophoresis to separate cross-linked from un-cross-linked RNA.

Activity Measurement. k_{cat}/K_m is the second-order rate constant for reaction of the ribozyme–guanosine complex with free RNA oligonucleotide substrate and has been shown to correspond to the rate constant of association of the substrate.[13] The measurements of k_{cat}/K_m were performed under the same conditions as those for the photo-cross-linking. All reactions were single turnover, with an excess of ribozyme (10 nM) and trace amount of oligonucleotide substrate (~1 nM). Reactions were initiated by addition of the 5′-end [^{32}P]-labeled oligonucleotide substrate (^{32}P–GGCCCUCUAAAAA) after 15 min preincubation of L-21 *Sca*I RNA in buffers described in Photo-cross-linking. Typically eight portions of 1 μL were removed from a 20-μL reaction mixture at specified times and quenched with 4 volumes of 40 mM EDTA in 90% formamide with 0.01% each bromophenol blue and xylene cyanol, 1 mM Tris, pH 7.5. Product and substrate were separated by electrophoresis on 20% polyacrylamide–7 M urea gels, and their ratio at each time point was quantitated using a PhosphorImager (Molecular Dynamics). Reactions were followed for 5 half-lifetimes, within which the disappearance of the substrate was first order, with an end point of ~5% corresponding to the unreactive starting material.

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