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# Direct measurement of oligonucleotide substrate binding to wild-type and mutant ribozymes from *Tetrahymena*

(RNA catalysis/gel mobility shift/binding energy/$Mg^{2+}$ dependence/dissociation constants)

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**ABSTRACT** **Like protein enzymes, RNA enzymes (ribozymes) provide specific binding sites for their substrates. We now show that equilibrium dissociation constants for complexes between the *Tetrahymena* ribozyme and its RNA substrates and products can be directly measured by electrophoresis in polyacrylamide gels containing divalent cations. Binding is $10^3$- to $10^4$-fold tighter (4–5 kcal/mol at 42°C) than expected from base-pairing interactions alone, implying that tertiary interactions also contribute to energetic stabilization. Binding decreases with single base changes in the substrate, substitution of deoxyribose sugars, and lower $Mg^{2+}$ concentration. $Ca^{2+}$, which enables the ribozyme to fold but is unable to mediate efficient RNA cleavage, promotes weaker substrate binding than $Mg^{2+}$. This indicates that $Mg^{2+}$ has special roles in both substrate binding and catalysis. Mutagenesis of a region near the internal guide sequence disrupts substrate binding, whereas binding is not significantly affected by a mutation of the guanosine-binding site. This approach should be generally useful for analysis of ribozyme variants independent of their catalytic activities.**

Ribozymes derived from the self-splicing intervening sequence (IVS) of *Tetrahymena* rRNA catalyze phosphodiester bond cleavage of oligonucleotide substrates (1, 2). The mechanism for this reaction is similar to the first step in self-splicing of the pre-rRNA (3). Substrate recognition is mediated by base-pairing interactions between an internal guide sequence (IGS) within the ribozyme and a complementary substrate (4–6). A schematic of this interaction is shown in Fig. 1. Given this model, one should be able to estimate the interaction energy by using rules developed for prediction of duplex stability (7–9). For a circular form of the same IVS, however, kinetic results suggest that oligonucleotides may bind to the active site with a greater affinity than expected for simple duplex formation (10, 11).

Nondenaturing gel electrophoresis has been extensively used for studying nucleic acid–protein associations (12–17). Complexes between RNA moieties have also been observed in native gels (18–20). Here we show that ribozyme–substrate complexes are stable in nondenaturing polyacrylamide gels containing $Mg^{2+}$ or $Ca^{2+}$. The ability to partition free and ribozyme-bound RNA substrate makes it possible to quantitatively study factors contributing to the energetics of association.

## METHODS

**RNA Preparation.** The L-21 *Sca* I ribozyme used in this study is a shortened form of the *Tetrahymena thermophila* pre-rRNA IVS, lacking the first 21 and last 5 nucleotides (the 3′ end being determined by a *Sca* I site in the DNA template). This ribozyme is 388 nucleotides in length, and $G^{414}$ has been deleted in order to study reactions in the presence of exogenous guanosine. The preparation of this ribozyme and its oligonucleotide substrates has been described (2). Oligonucleotide products were transcribed from the same DNA templates as the substrates, but ATP was omitted to cause the phage T7 RNA polymerase to terminate before transcription of the substrate adenine residues. The carrier oligonucleotide of unrelated sequence (5′-GCGGUAGGUUGCCCN-3′, where N is a heterogeneous terminal nucleotide) and carrier tRNA were also prepared by transcription with T7 RNA polymerase and were gifts from M. Fedor and E. Tinkle (University of Colorado, Boulder, CO).

**Direct Determination of Binding Constants.** Ribozyme dilutions at 2× final concentration were preincubated at 50°C for 10 min before mixing with equal volumes of 1.0 nM oligonucleotide substrate (S) or product (P). The oligonucleotide was 5′ end-labeled by treatment with calf intestinal phosphatase followed by polynucleotide kinase and [$\gamma$-$^{32}$P]-ATP (2). Samples were incubated at the indicated temperatures for 5 min in 50 mM Tris, pH 7.5/0.1 mM EDTA/10 mM NaCl/10 mM $MgCl_2$/3% glycerol/0.05% xylene cyanol and then loaded immediately on a polyacrylamide gel (0.4 mm × 230 mm × 280 mm) running at 10 W. Binding was unaffected by the presence of the dye or glycerol in the mixture (as determined by substitution with 5% sucrose). The electrophoresis buffer was 100 mM Tris–Hepes, pH 7.5/0.1 mM EDTA/10 mM $Mg^{2+}$. In addition to electrophoresis buffer, the 10% polyacrylamide gel was prepared with a 30:1 weight ratio of acrylamide to *N*,*N*′-methylenebisacrylamide. Gels were run at constant temperature by clamping to a reservoir fitted with recirculating coils. Quantitation of free oligonucleotide and its complex was performed on an AMBIS radioanalytic scanner. Except where noted, binding of substrate and product oligonucleotides was studied in the absence of guanosine, in order to prevent strand scission. Additional controls were performed to ensure that the guanosine-independent hydrolysis reaction (21) did not cleave significant amounts of the substrate under the conditions of the binding study. The dissociation constant ($K_d$) obtained using different ribozyme preparations varied from 0.7 to 1.3 nM for GGCCCUCU. Similar day-to-day variation in $K_d$ was observed using different IVS dilutions of the same ribozyme preparation. Studies of oligonucleotide binding in the presence of guanosine were performed at room temperature under the conditions described above except that the electrophoresis buffer was 50 mM Tris/50 mM borate/0.1 mM EDTA, pH 8.3/0.5 mM guanosine/10 mM $MgCl_2$.

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Abbreviations: IVS, intervening sequence; IGS, internal guide sequence.

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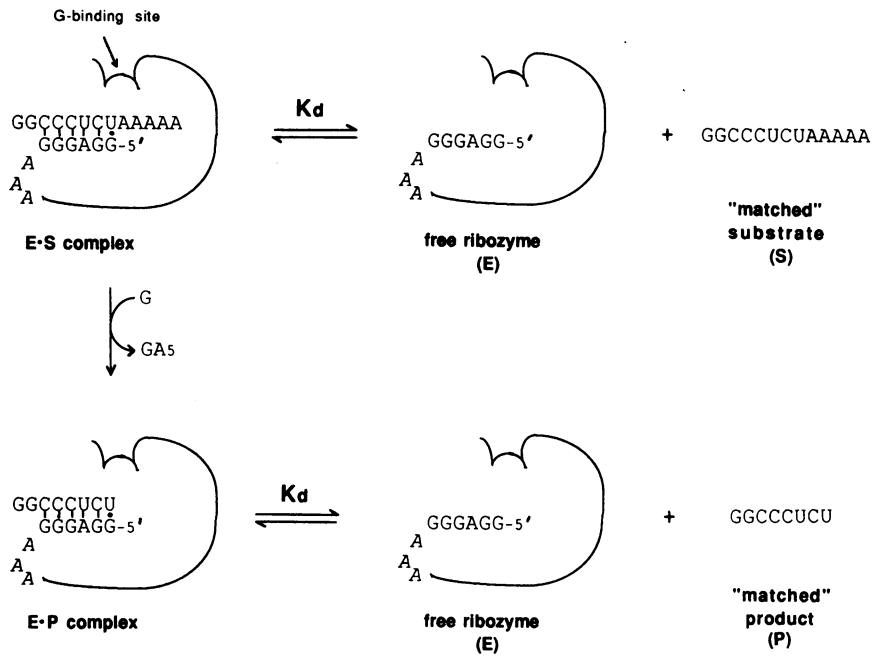


FIG. 1. Schematic of the interaction between the L-21 *Sca* I ribozyme and its "matched" substrate and product. In the E·S complex, base-pairing joins the substrate to a portion of the IGS (3'-GGGAGG-5') to form helix P1. After ribozyme-catalyzed cleavage, similar interactions persist in the E·P complex. The guanosine-binding site is depicted above the P1 helix, where it is poised to effect scission of the substrate U-A bond. Two of the three italicized adenine residues are deleted in the J1/2–2A variant of the ribozyme.

**Determination of Binding by Competition.** The association of GGCCCGCU, GGCCCGCUA$_5$, and d(GGCCCUCU) to the ribozyme was studied by competition with the radiolabeled probe $^{32}$P-GGCCCUCU. The validity of the competition assay was verified in two ways. Unlabeled GGCCCUCU was found to compete with $^{32}$P-GGCCCUCU in binding to the ribozyme at 25°C. A calculated $K_d$ for the inhibitor was found to be 1.5 nM. Second, the binding of $^{32}$P-GGCCCGCU was studied directly at several temperatures and found to be only a factor of 3 weaker than that determined by competition. The competitive $K_d$ determination is more reliable due to smearing of the relatively unstable $^{32}$P-GGCCCGCU–ribozyme complex in the gel, which artifically lowers the observed $K_d$.

**Free Energy Calculations.** The observed free energies were determined as $\Delta G^\circ = -RT \ln(1/K_d)$, where $R = 0.00198$ kcal·mol$^{-1}$·K$^{-1}$ and $T$ is in kelvins. The calculated free energies were determined by $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ using $\Delta H^\circ$ and $\Delta S^\circ$ values from refs. 8 and 9. The terminal A–G mismatch and the terminal G–U pair were included in the calculation.

## RESULTS

**Method for Direct Determination of Binding Constants.** The oligonucleotide GGCCCUCU, which forms a "matched" duplex with the guide sequence of the ribozyme, represents one of the products of ribozyme-catalyzed cleavage of RNA (Fig. 1). The binding of this product to the L-21 *Sca* I ribozyme in 10 mM $MgCl_2$ is illustrated in Fig. 2*A*. The amount of oligonucleotide bound increases with increasing concentration of ribozyme in a manner that gives a good fit to a theoretical binding curve, as shown in Fig. 2*B*. The extent of binding was independent of incubation time (from 1 to 10 min) even at the lowest ribozyme concentrations used and was thereby judged to be at equilibrium. Binding required a divalent cation, but binding in 10 mM $MgCl_2$ was not significantly different from that in 10 mM $Mg(OAc)_2$. Binding was unaffected by other experimental variables such as the percentage of polyacrylamide in the gel (5–15%), the concentration of substrate (0.05–1 nM), the volume of sample loaded on the gel, or the electrophoresis time. Carrier RNA molecules, including tRNA and an unstructured oligonucleotide of unrelated sequence (both at 400 nM in nucleotides), did not change the $K_d$ of CCCUCU and therefore did not compete for the binding site. This confirms that complementarity between the active site and oligonucleotides is essential for association.

**Equilibrium Dissociation Constants of Oligonucleotide–Ribozyme Complexes.** The dissociation constant of GGCCCUCU obtained by inspection of the gel midpoint (Fig. 2*A*) or the fit to a theoretical binding curve (Fig. 2*B*) is ≈1 nM at 25°C and 42°C. This value corresponds to a binding free energy of −12.2 (25°C) or −12.9 (42°C) kcal/mol, which is ≈0.4 kcal/mol greater than the energy calculated by considering base-pairing alone at 25°C and 4.0 kcal/mol greater at 42°C.

The structure of RNA substrates and products was varied in order to learn more about specific binding requirements. A single base substitution at a position three nucleotides from the 3' end of the product adversely affects binding to the ribozyme. This is shown by comparison of **1** with **4** and **2** with **5** in Table 1. However, the binding remains tighter than that calculated for a simple mismatched helix, 4.5 kcal/mol of extra binding energy at 25°C, and 5.5 kcal/mol at 42°C for **4**. There is little difference in the $K_d$ of reaction substrates and their corresponding products. The substrate **2** binds with almost the same affinity as the corresponding product **1**, whereas mismatched **5** binds consistently more poorly than the product **4**. Nevertheless, it is clear for both matched and mismatched RNAs that the A$_5$ moiety makes little net contribution to binding, consistent with the simple picture in Fig. 1. Binding of the mismatched product **4** and wild-type product **3** is temperature-dependent, whereas that of the matched product **1** is almost independent of temperature over the range studied. Therefore, differences between the various substrates become larger at higher temperatures.

The ribozyme contains two binding sites: one for the RNA substrate and one for a guanosine substrate. It is conceivable that binding of one substrate could be affected by binding of

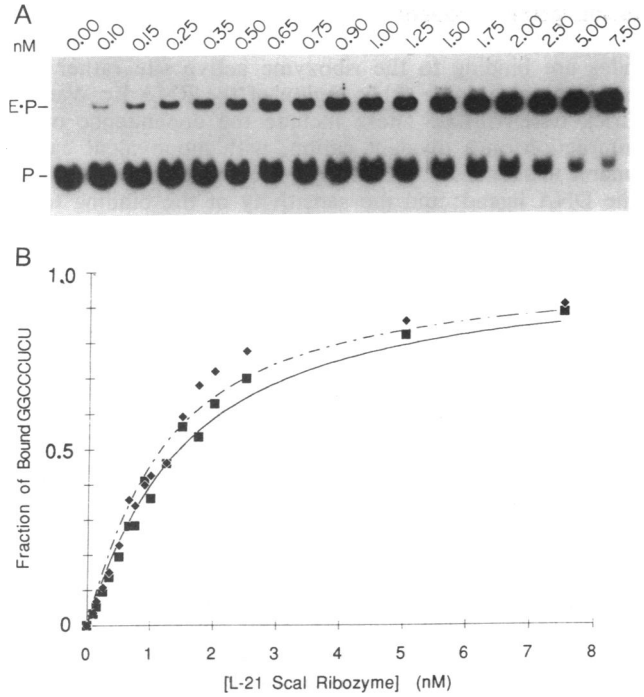


FIG. 2. Equilibrium binding of GGCCCUCU to the L-21 *Sca* I ribozyme in 10 mM $MgCl_2$. (*A*) Autoradiograph of $^{32}$P-GGCCCUCU (P, 0.5 nM) binding to increasing amounts of ribozyme (E) at 25°C. The concentration of ribozyme is indicated above each lane. Lanes correspond to the data shown in *B*. The midpoint of the experiment occurs between lanes where [E] is 1.25 and 1.5 nM, respectively. Solving the equilibrium equation at the midpoint where $[P_{free}] = [P_{bound}]$, $K_d = [E_{total\ at\ midpoint}] - \frac{1}{2}[P_{total}]$. $K_d$ by this method is therefore 1.0–1.25 nM. (*B*) Binding of P to the L-21 *Sca* I ribozyme at 42°C (■) and 25°C (◆). The solid and broken lines represent the best fits of the data to ideal binding curves with $K_d$ values of 1.2 nM ($S_y = \pm 7\%$) and 0.9 nM ($S_y = \pm 9\%$), respectively; the relative standard error of estimate $(S_y) = 100[\Sigma(F_T - F_E)^2/n]^{1/2}/\langle F_E \rangle$, where $F_T$ and $F_E$ are the fractions bound as determined from theory and experiment, respectively, and $n$ is the number of data points.

the other. However, binding of the oligonucleotides CCCUCU, CUCUCU, and CCCGCU is unaffected by high concentrations of guanosine (500 $\mu$M) in the incubation buffer and in the native gel itself. Preliminary results indicate that the $K_d$ for guanosine binding to the ribozyme is 200 ± 100 $\mu$M under similar conditions (P. Legault, D. Herschlag, and T.R.C., unpublished results). Therefore, the ribozyme is expected to be more than half-saturated with guanosine in the product-binding experiments.

Table 1. Binding of oligonucleotides to the ribozyme as a function of sequence and structure

| Oligonucleotide | | $K_d$, nM | |
|---|---|---|---|
| No. | Sequence | 25°C | 42°C |
| **1** | GGCCCUCU | 0.9 | 1.2 |
| **2** | $GGCCCUCUA_5$ | 1.0 | 1.3 |
| **3** | CUCUCU* | 1.6 | 22 |
| **4** | GGCCCGCU | 8.5 | 100 |
| **5** | $GGCCCGCUA_5$ | 35 | 130 |
| **6** | d(GGCCCUCU) | 65 | 2100 |

Data for binding of GGCCCGCU (200 nM), $GGCCCGCUA_5$ (200 nM), and d(GGCCCUCU) (1 $\mu$M) were obtained by competitive inhibition of GGCCCUCU (0.5 nM) binding at the indicated temperatures.

*The wild-type sequence studied in this set of experiments had the two guanine residues at its 5′ end removed by T1 RNA endonuclease (RNase T1). The presence of these two guanine residues (which are needed to obtain efficient transcription by phage T7 RNA polymerase) does not significantly affect binding of either the CCCUCU or the CUCUCU oligonucleotides (data not shown).

The data in Table 1 also describe the binding of DNA. The DNA product **6** binds with considerably less affinity than its RNA counterpart **1**, in agreement with results reported recently (22). Therefore, binding is greatly affected by the lack of 2′-hydroxyl groups or by structural constraints imposed by an RNA·DNA hybrid within the ribozyme–substrate complex. The DNA product binds with considerable temperature dependence, as expected for a binding interaction based primarily upon base-pairing. This indicates that the thermodynamic parameters governing association of DNA to the ribozyme are different than those describing the binding of the RNA analogue of this sequence. The thermodynamics of ribozyme–substrate association will be addressed in more detail in a subsequent manuscript.

**Divalent Cation Requirements.** $Mg^{2+}$ has a pronounced effect on the binding of GGCCCUCU, GGCCCGCU, and d(GGCCCUCU) to the ribozyme. Model studies show that the free energy of association of complementary oligonucleotides is similar at 50 mM $Na^+$ and at 2 mM $Mg^{2+}$ (23). However, while binding of GGCCCUCU to the ribozyme occurred at $Mg^{2+}$ concentrations as low as 1 mM (Fig. 3), no binding was observed in either 10 mM $Na^+$ or 50 mM $Na^+$ in the absence of $Mg^{2+}$ at 25°C.

$Mg^{2+}$ dependence of binding appears to increase in the order GGCCCUCU < GGCCCGCU < d(GGCCCUCU), as shown in Fig. 3. Plots of log $K_d$ vs. log $[Mg^{2+}]$ have slopes that differ by a factor of 2. The slopes of these lines represent the minimum number of additional $Mg^{2+}$ ions bound upon formation of the ribozyme–product complex beyond those bound in free ribozyme and free product. The log/log plot shown in Fig. 3 is similar to that described by Wyman (24) for site-binding of ligands to a macromolecule or that described by Record *et al.* (ref. 25; see also ref. 26) for the number of ions liberated upon helix dissociation. Binding of GGCCCUCU involves the uptake of at least one additional $Mg^{2+}$,

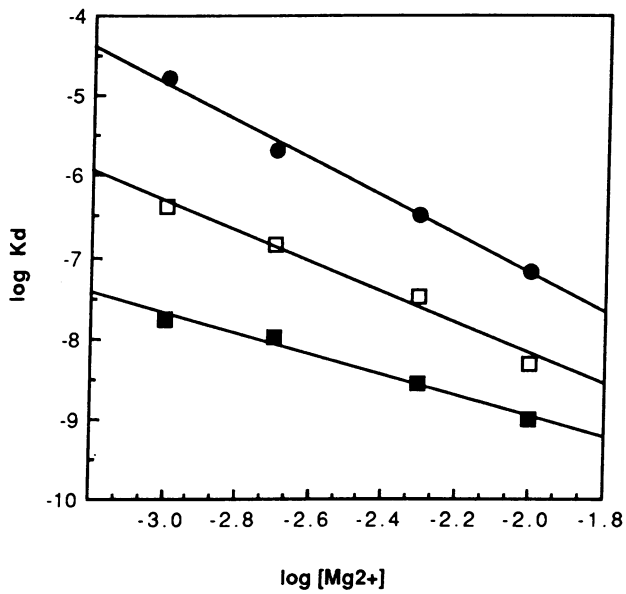


FIG. 3. $Mg^{2+}$ dependence of $K_d$ for the matched oligonucleotide **1** (■), the mismatched oligonucleotide **4** (□), and the DNA analogue of the matched oligonucleotide **6** (●). Slopes of the lines are 1.26, 1.87, and 2.36, respectively. The equation describing the lines, observed $\log K_d^{E\cdot P} = \log K_d^{E\cdot P\cdot Mg} - n(\log[Mg^{2+}_{total}])$, was derived from the equilibrium equation $E + P + nMg^{2+} \rightleftarrows E\cdot P\cdot nMg^{2+}$ by assuming that only a small fraction of the total $Mg^{2+}$ is bound and that no free E·P forms in the absence of $Mg^{2+}$.

while that of GGCCCGCU or d(GGCCCUCU) involves uptake of at least two additional $Mg^{2+}$. Therefore, binding of the more poorly matched products requires more $Mg^{2+}$, possibly in order to overcome structural impediments to association with the ribozyme.

It is known from both activity and structural studies that $Ca^{2+}$ is able to promote ribozyme folding similar to that promoted by $Mg^{2+}$ (refs. 27 and 28; D. Celander and T.R.C., unpublished work). The gel electrophoretic method was used to determine whether the $Ca^{2+}$ form of the ribozyme is competent for substrate binding. Under conditions of 10 mM $Ca^{2+}$ at 25°C, GGCCCUCU is able to bind to the ribozyme with a $K_d$ of 39 ± 2 nM. (The error in this measurement is based upon multiple titrations.) This represents considerably weaker binding than that observed with $Mg^{2+}$ at the same concentrations and it approaches that observed for the $Mg^{2+}$ form of the ribozyme with the DNA substrate. Nevertheless, under conditions where substrate is bound to the $Ca^{2+}$ form of the ribozyme ([E] >> $K_d$ in $Ca^{2+}$), little, if any, cleavage occurs. [Cleavage rate is $< 5 \times 10^{-6}$ $min^{-1}$ (A.M.P. and T.R.C., unpublished results).] Therefore, ribozymes substituted with $Ca^{2+}$ can fold and bind substrate, but they are highly defective in their ability to undergo the chemical steps involved in cleavage of the U-A bond of substrate.

**Mutant Ribozymes.** As described above, tight substrate association appears to be dependent on structural constraints imposed by the ribozyme active site. In order to examine the effects of ribozyme structure and sequence, two mutants of the L-21 *Sca* I ribozyme were studied. The G264A:C311U double mutant contains a mutation of the guanosine-binding site within the core of the ribozyme, as demonstrated by Michel *et al.* (29), and is deficient in guanosine-dependent substrate cleavage (P. Legault, D. Herschlag, and T.R.C., unpublished results). As shown in Table 2, the core mutation does not significantly affect binding of the RNA product or substrate. Deleterious effects on activity of the G264A:C311U mutant are not due to any perturbation of RNA substrate binding. The J1/2–2A mutant is missing two of the three adenine residues (italicized in Fig. 1) that join the IGS to the remainder of the ribozyme. Substrate–ribozyme interactions are highly perturbed in this mutant (Table 2), demonstrating that sequences near the substrate-binding site can greatly affect binding. Nevertheless, the J1/2–2A mutant is catalytically active; in fact, the J1/2–2A mutant is a "better ribozyme" under saturating substrate conditions, because the weaker association of the product accelerates the rate-limiting product-release step (B. Young, D. Herschlag, and T.R.C., unpublished work).

## DISCUSSION

We have shown that the association of oligonucleotides with the *Tetrahymena* ribozyme can be monitored by nondenaturing gel electrophoresis. Several characteristics of the association provide a strong argument that the oligonucleotides are binding to the ribozyme active site rather than hybridizing to some other region of the RNA by Watson–Crick base-pairing. These include the dependence of the interaction on a divalent cation, with monovalent cations unable to substitute; the strong preference for the RNA over the DNA ligand; and the sensitivity of the binding to the deletion of two adenine residues adjacent to but not within the substrate-binding site. Furthermore, for the RNA products and substrates, the magnitude of the binding constant at 42°C is ≈4 kcal/mol larger than calculated from base-pairing alone, and the lack of temperature dependence in binding is not that expected for simple duplex formation. We ascribe these characteristics to the presence of tertiary interactions between the RNA oligonucleotides and the ribozyme. It is important to note, however, that the calculated values which form the basis for these comparisons are derived from measurements performed at temperatures and ionic conditions different from those used here. Thus, it is possible that some of the difference between calculated and observed $\Delta G^\circ$ values is due to unexpected thermodynamic behavior of helix formation rather than tertiary interactions with the ribozyme.

Equilibrium dissociation constants determined by the gel electrophoretic method, when extrapolated to 50°C, are in reasonable agreement with values determined by studying the kinetics of the cleavage reaction at 50°C under similar ionic conditions (10 mM $Mg^{2+}$) (21). Direct measurement of binding of a fluorescent oligonucleotide to the circular form of the *Tetrahymena* IVS RNA has been reported using fluorescence quenching; similar conclusions were reached regarding the extra stabilization of the complex (30). However, differences in the oligonucleotide, the form of the ribozyme, and the temperature preclude direct comparison of the binding constant with those obtained in the present study.

The data indicate that binding is specifically stabilized by $Mg^{2+}$, presumably mediated by tertiary interactions between the ribozyme core and the IGS-associated substrate. The perturbation of binding affinity observed upon substitution of deoxyribose sugars within the substrate may indicate that 2′-hydroxyl groups are directly involved in $Mg^{2+}$ coordination (31), although it is possible that structural differences between RNA·RNA and RNA·DNA duplexes indirectly affect binding. The data presented here do not permit differentiation between $Mg^{2+}$ bound to specific sites and an electrostatic model in which fully hydrated $Mg^{2+}$ participates through more diffuse ionic effects. In either case, at least one $Mg^{2+}$ is taken up in forming the GGCCCUCU–ribozyme complex, while more interacting $Mg^{2+}$ may be required to stabilize ribozyme binding of poorly matched and DNA products.

Extrapolation of the graphs of Fig. 3 to high $Mg^{2+}$ concentration gives approximate convergence of the $K_d$ values at 0.01 nM. Such convergence would indicate that the intrinsic differences in $K_d$ between the various oligonucleotides are minimal and that the differences apparent at physiological $Mg^{2+}$ concentrations are largely due to different amounts of $Mg^{2+}$ taken up during binding. In the absence of additional data, however, it is equally tenable that the graphs would plateau at higher $Mg^{2+}$ concentration.

Ribozyme–product association is weaker in the presence of $Ca^{2+}$, suggesting that this ion fails to mediate the tertiary interactions promoted by $Mg^{2+}$. Nonetheless, $Ca^{2+}$ is able to promote formation of a ribozyme–substrate complex and therefore may fail to promote catalytic activity at a chemical step, rather than a substrate binding step. From this and other studies it is now possible to define three distinct roles for divalent cations in the reaction of the *Tetrahymena* ribozyme with its substrates. (*i*) They are involved in folding of the ribozyme into a tertiary structure, $Mg^{2+}$ and $Ca^{2+}$ promoting

Table 2. Binding affinity of L-21 *Sca* I ribozyme mutants at 42°C and 10 mM $Mg^{2+}$

| Form of L-21 *Sca* I RNA | $K_d$, nM | |
|---|---|---|
| | GGCCCUCU | GGCCCUCUA$_5$ |
| Wild type | 1.2 | 1.3 |
| G264A:C311U* | 1.4 | 2.2 |
| J1/2−2A† | 98 | 73 |

*A double mutation of the guanosine-binding site (29). $G^{264}$ is changed to an A and the C to which it is base-paired is changed to a U. These mutations were introduced into the L-21 *Sca* I RNA by oligonucleotide-directed mutagenesis and the phenotype observed in the splicing reaction was confirmed in this construct (P. Legault, D. Herschlag, and T.R.C., unpublished results).

†Lacks two of the three adenine residues from the junction between paired region P1 and the rest of the ribozyme (see Fig. 1).

the formation of very similar, although not identical structures (ref. 28; D. Celander and T.R.C., unpublished work). (*ii*) They are involved in substrate binding ($Mg^{2+} >> Ca^{2+}$). (*iii*) They have some more direct role in catalysis ($Mg^{2+}$ or $Mn^{2+}$) (27).

Although $Ca^{2+}$ and $Mg^{2+}$ are both group IIA divalent metal ions, which prefer oxygen ligands, several important characteristics differentiate the ions and explain why they do not necessarily perform the same structural roles in biological systems (32, 33). The two ions have different radii (0.99 Å for $Ca^{2+}$ and 0.80 Å for $Mg^{2+}$; ref. 34) and different charge densities, and they may have different relative preferences for -OH, $-O^-$, or even N ligands. Perhaps most important, the ions have different optimum coordination geometries. The most common geometry for $Mg^{2+}$ is six to seven (35), while that of $Ca^{2+}$ is eight (36). However, coordination about $Ca^{2+}$ is considerably more fluxional, $Ca^{2+}$-binding sites of six, seven, and eight ligands having been observed (36–38). Given that there are a multiplicity of different site sizes and coordination environments in a large RNA molecule (39–41), it is not surprising that $Mg^{2+}$ and $Ca^{2+}$ behave differently in binding substrate.

The gel binding assay provides a means for examining ribozymes that are inactive in the presence of certain metal ions or inactivated by mutations. Mutational analysis of the ribozyme shows that RNA substrate binding is disrupted by a mutation near the IGS, but not by one within the guanosine-binding site. Furthermore, presence of saturating guanosine does not perturb the $K_d$ for binding of oligonucleotides. These results confirm that different domains of the ribozyme are able to act independently when binding the two substrates (see also ref. 21).

Nondenaturing polyacrylamide gel electrophoresis provides a rapid, convenient means for exploring the substrate binding event independently and directly. Although $K_d$ values can be inferred from kinetic measurements, that approach can be complex, especially when $K_d$ is not equal to $K_m$ (21). In addition, one can use the electrophoretic method to probe the substrate recognition of mutants and defective ribozymes lacking catalytic activity. This approach should facilitate the identification of sequences and structures involved in substrate binding in this and other ribozymes.

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