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Activity, Folding and Z-DNA Formation of the 8-17 DNAzyme in the Presence of Monovalent Ions

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Abstract: The effect of monovalent ions on both the reactivity and global folding of the 8-17 DNAzyme is investigated, and the results are compared with those of the hammerhead ribozyme, which has similar size and secondary structure. In contrast to the hammerhead ribozyme, the 8-17 DNAzyme activity is not detectable in the presence of 4 M K^+, Rb^+, or Cs^+ or in the presence of 80 mM, $[Co(NH_3)_6]^{3+}$. Only 4 M Li^+, NH_4^+ and, to a lesser extent, Na^+ conferred detectable activity. The observed rate constants ($k_{obs} \approx 10^{-3}$ min^{-1} for Li^+ and NH_4^+) are ~1000-fold lower than that in the presence of 10 mM Mg^{2+}, and ~200,000-fold slower than that in the presence of 100 μM Pb^{2+}. Since the hammerhead ribozyme displays monovalent ion-dependent activity that is often within ~10-fold of divalent metal ion-dependent activity, these results suggest that the 8-17 DNAzyme, obtained by *in vitro* selections, has evolved to have a more stringent divalent metal ion requirement for high activity as compared to the naturally occurring ribozymes, making the 8-17 DNAzyme an excellent choice as a Pb^{2+} sensor with high selectivity. In contrast to the activity data, folding was observed in the presence of all the monovalent ions investigated, although those monovalent ions that do not support DNAzyme activity have weaker binding affinity ($K_d \sim 0.35$ M for Rb^+ and Cs^+), while those that confer DNAzyme activity possess stronger affinity ($K_d \sim 0.22$ M for Li^+, Na^+ and NH_4^+). In addition, a correlation between metal ion charge density, binding affinity and enzyme activity was found among mono- and divalent metal ions except Pb^{2+}; higher charge density resulted in stronger affinity and higher activity, suggesting that the observed folding and activity is at least partially due to electrostatic interactions between ions and the DNAzyme. Finally, circular dichroism (CD) study has revealed Z-DNA formation with the monovalent metal ions, Zn^{2+} and Mg^{2+}; the K_d values obtained using CD were in the same range as those obtained from folding studies using FRET. However, Z-DNA formation was not observed with Pb^{2+}. These results indicate that Pb^{2+}-dependent function follows a different mechanism from the monovalent metal ions and other divalent metal ions; in the presence of latter metal ions, metal-ion dependent folding and structural changes, including formation of Z-DNA, play an important role in the catalytic function of the 8-17 DNAzyme.

Introduction

Metal and nucleic acid interaction has been a fascinating field of study with potential for advancement of both basic coordination chemistry and structural biology, and practical biotechnological and pharmaceutical applications.[1−11] This field became even more exciting when RNA[12,13] and then DNA[14] were discovered in the early 1980s and 1990s, respectively, to possess catalytic or enzymatic functions, supported by metal ion cofactors. The catalytic RNA, often called ribozymes, occur naturally, whereas catalytic DNA (often called deoxyribozymes or DNAzymes) have not yet been isolated in nature, and they are obtained in the laboratory by a combinatorial biology technique called *in vitro* selection.[14−18]

These nucleic acid enzymes are efficient catalysts in the presence of divalent metal ion cofactors that have been proposed

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to play both structural and catalytic roles, i.e., they can function to stabilize the active structure of the enzyme and can also be directly involved in active-site chemistry.[6,10] Since their discovery, these nucleic acid enzymes have been mainly studied with respect to the divalent metal-dependent activity and folding.[6,7,19−32] Despite progresses, the exact role of metal ions in the nucleic acid enzymes, particularly in DNAzymes, is still far less understood as compared to that in metalloprotein enzymes. A number of protein enzymes that utilize monovalent ions are known,[33,34] and high concentrations of monovalent ions have been known to replace divalent ions in simple nucleic acid chemistry, such as hydrolysis of ATP.[35] More interestingly, although divalent metal ions are the most efficient cofactors for catalysis in nucleic acid enzymes, recent studies suggest that the naturally occurring ribozymes such as the hairpin,[36−40] hammerhead,[39,41,42] hepatitis delta virus (HDV),[43,44] Varkud satellite (VS),[39] and glmS ribozymes[45] as well as some *in vitro* selected ribozymes and DNAzymes[46] can promote catalysis in the presence of monovalent ions, such as Li^+, Na^+, and K^+. Monovalent metal ion-dependent folding of small ribozymes has also been studied, and it is interesting to note that these ions have shown their capability in inducing the similar active conformation as seen with divalent metal ions.[44,47−53] These findings have been usually interpreted to mean that these ribozymes require metal ions mainly for electrostatic or structural stabilization, while for chemical catalysis, their role is minimal or absent, even though the conditions of the study are far from physiological conditions.

While a number of studies have been reported on ribozyme catalysis in the presence of monovalent ions alone, there have not been similar reports on the monovalent ion dependent-activity of catalytically proficient DNAzymes that have been selected in the presence of divalent ions. Geyer et al. reported the *in vitro* selection for an RNA-cleaving DNAzyme which was carried out in the presence of Na^+ without any divalent metal ions in the buffer.[46] This study resulted in DNAzymes that were active without any divalent metal ion and catalysis was supported by monovalent ions only. Interestingly, the addition of divalent metal ions did not change the catalytic rate significantly. Thus, this DNAzyme may be fundamentally different from the naturally occurring ribozymes and other *in vitro* selected DNAzymes that utilize divalent metal ions as their most efficient cofactor. Therefore, it is important to study the effects of monovalent ions on DNAzymes that are active in the presence of divalent metal ions. Comparison of such effects on DNAzymes with those on ribozymes will be very interesting.

We chose to study the 8-17 DNAzyme[54−61] in the presence of monovalent ions alone in order to investigate whether monovalent metal ions are required for global folding and catalysis. The 8-17 DNAzyme is a well studied DNAzyme that catalyzes the cleavage of a DNA substrate containing a single RNA base at the cleavage site (Figure 1a). It is active in the presence of divalent metal ions in the following order: $Pb^{2+} \gg Zn^{2+} \gg Cd^{2+} \gg Mg^{2+} \sim Ca^{2+}$.[62] Due to its high specificity for Pb^{2+}, the 8-17 DNAzyme has been converted into fluorescent,[63,64] colorimetric[65−68] and electrochemical sensors[69] for Pb^{2+}. The divalent metal ion-dependent folding and cleavage of the 8-17 DNAzyme has been studied using fluorescence resonance energy transfer (FRET) which suggests that the 8-17 DNAzyme folds

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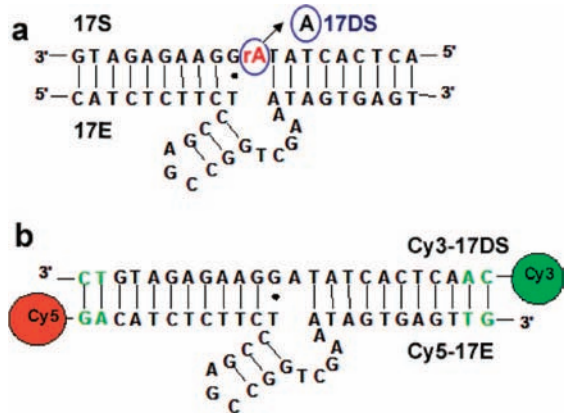

Figure 1. Predicted secondary structures of the 8-17 DNAzyme (a) The 8-17 DNAzyme used for activity assays. The substrate containing a single RNA base, rA (indicated in red) at the cleavage site is called 17S and the enzyme is called 17E. For the CD studies, the ribonucleotide (rA) at the cleavage site on the substrate was replaced with a deoxyribonucleotide (A) to render it noncleavable, and this was called 17DS. (b) Modified 8-17 DNAzyme used for FRET study. The end of each arm was extended by two base pairs for thermal stabilization, and the rA was replaced with A. Substrate (Cy3-17DS) and enzyme (Cy5-17E) were modified with Cy3 and Cy5 fluorophores, respectively to form a FRET pair.

into a compact structure, followed by cleavage in the presence of Zn^{2+} and Mg^{2+}.[70−73] In the presence of Pb^{2+}, which is the most efficient cofactor, conformational change was not observed prior to cleavage suggesting that the DNAzyme may use two different modes of activation; the DNAzyme may be prearranged to accept Pb^{2+} as the cofactor, whereas with the other metal ions a metal induced global folding step precedes catalysis.[71] However, no monovalent ion dependence of the 8-17 DNAzyme has been reported. In addition to fundamental insights into its metal ion-dependent folding and activity, investigation of monovalent ion dependence of the 8-17 DNAzyme will also help design more selective sensors because monovalent ions are often present during sensing applications. Therefore, herein we report the activity and folding studies on the 8-17 DNAzyme in the presence of monovalent ions. We have found clear differences in monovalent ion dependence between the DNAzyme and ribozymes and also observed close correlation between monovalent ion-dependent activities and folding probed via FRET. More interestingly, we have used circular dichroism (CD) to find evidence for Z-DNA formation of the 8-17 DNAzyme in the presence of monovalent ions and Mg^{2+} and Zn^{2+}. However, the same structural changes are not observed in the presence of Pb^{2+}. The significance of these findings in elucidation of metal ion-dependent structure and function of the 8-17 DNAzyme is discussed.

Materials and Methods

All oligonucleotides (HPLC purified) were purchased from Integrated DNA Technologies Inc. Ultrapure salts (99.999%), LiCl, NaCl, KCl, RbCl, CsCl and NH_4Cl were purchased from Alfa Aezer.

Bulk Activity Assays. The activity assays were carried out in a reaction buffer containing 50 mM Na-HEPES acetate (pH 7.5) and 25 mM EDTA. The EDTA was added to avoid any divalent metal ion-dependent activity. Monovalent metal ion solutions (5 M) were prepared in the reaction buffer, and the pH was readjusted to 7.5 using a glass pH electrode (Orion) and verified using a pH paper. The substrate 17S was 5′-labeled with γ-^{32}P dATP (GE Healthcare) using T4 kinase from Invitrogen. In previous studies, divalent metal ion-dependent reactions were initiated by mixing equal volumes of 2× concentration of metal solution to 2× concentration of enzyme−substrate solution to obtain the desired concentration (1×) of all components.[62] Since some monovalent ions could not be prepared at a concentration greater than 5 M, a modified protocol, as outlined below, was used for these single turnover activity assays. Enzyme (5 × 5 μM) and ^{32}P-labeled substrate (5 × 1 nM) were annealed by heating the mixture at 90 °C for 3 min and cooling to room temperature over 30 min. The reaction was initiated by adding one volume of enzyme−substrate mixture (5 × concentration) into 4 volumes of 5 M metal solution at room temperature, such that the final concentrations of the enzyme, substrate, and metal ion were 5 μM, 1 nM, and 4 M, respectively. It was ensured that starting the reaction with a 5× concentration of enzyme−substrate solution, instead of 2× concentration did not produce any changes in the reaction kinetics (see Supporting Information, S1). The reaction was stopped at designed time points by adding a 2.5 μL aliquot of the reaction mixture into 50 μL of stop buffer containing 8 M urea + 50 mM EDTA + 0.05% bromophenol blue + 0.05% xylene cyanol. Divalent metal-dependent reactions can be quenched by chelating the metal with EDTA in the stop buffer;[62] however, monovalent metal ions are not effectively chelated using EDTA. Therefore, a large volume (20×) of stop solution was used for these experiments as this would stop the reaction by diluting the ion concentration significantly. The effectiveness of quenching the reaction by this method has been verified (data not shown).

Cleaved products and uncleaved substrate were separated by 20% polyacrylamide gel electrophoresis and quantified with a Molecular Dynamics Storm 430 phosphorimager. The percentage of cleavage was analyzed using Image Quant software (Molecular Dynamics). Kinetic curves were plotted using Sigma Plot and fit to the equation: $\%\ P_t = \%\ P_0 + \%\ P_\infty(1 - e^{-kt})$ where $\%\ P_0$ is the initial percent product ($t = 0$), $\%\ P_\infty$ is the % product at end point of the reaction ($t = \infty$), $\%\ P_t$ is the % product at any time t, and k is the observed rate of cleavage (called k_{obs}).

FRET experiments. To study metal-induced folding, 10 μM each of fluorophore-labeled substrate and enzyme, called Cy3-17DS and Cy5-17E respectively (Figure 1b) were annealed in 50 mM Na-HEPES acetate buffer (pH 7.5) by heating the sample at 85 °C for 5 min and subsequently cooling it to 4 °C over 2 h. The annealed product was purified with a native 16% polyacrylamide gel at 4 °C.[70,74] The band containing the annealed DNAzyme was located by inspection, cut out, crushed and subsequently recovered by soaking the crushed gel bits in 50 mM Na-HEPES acetate, pH 7.5. All the procedures were carried out at 4 °C to avoid dissociation of the DNAzyme complex. The sample concentration was determined by measuring its absorption at 260 nm, and the final concentration was diluted to <100 nM in the same buffer. At this concentration, the fluorophores were dilute enough to avoid inner-filter effects. Concentrated metal ion solutions in 50 mM Na-HEPES acetate, pH 7.5 were titrated into the sample to initiate folding.

Steady-state fluorescence emission spectra were recorded on a Fluromax-2 fluorometer (HORIBA Jobin Yvon Inc., NJ). Polarization artifacts were avoided by setting the polarizers under "magic angle conditions".[75] Fluorescence measurements were carried out at 10 °C to avoid the denaturation of the DNAzyme complex.

The FRET efficiency (E_{FRET}) was calculated using the $(ratio)_A$ method.[72] A detailed description of the $(ratio)_A$ method can be found in the literature.[76] Briefly, for a FRET pair, when the donor

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(D) is excited, the nearby (10−100 Å) acceptor (A) can receive energy from the donor with efficiency, $(ratio)_A$expressed as:[76]

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

where R is the donor-to-acceptor distance. R_0 is defined by

$$R_0^6 = 8.785 \times 10^{23} \times \Phi^D \times k^2 \times \eta^{-4} \times J(\upsilon) \text{Å}^6$$

Φ^D is the quantum yield of the donor and κ^2 is the orientation factor for dipole coupling. If both the donor and acceptor can rotate freely during the excited-state lifetime of the donor, κ^2 has the average value of 2/3.[77] η is the refractive index of the media. $J(\upsilon)$ is the overlap integral of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor.

In the $(ratio)_A$ method, the FRET spectrum $F(\lambda_{em},\lambda_{ex}^D)$ (at λ_{ex}^D, the acceptor may have some absorbance also) is fitted to the sum of the two components. The first component is the emission spectrum of the sample $F(\lambda_{em},\lambda_{ex}^A)$ (only the acceptor absorbs at λ_{ex}^A). The second component is a singly labeled donor emission spectrum $F^D(\lambda_{em},\lambda_{ex}^D)$ excited at λ_{ex}^D.

$$F(\lambda_{em},\lambda_{ex}^D) = (\text{ratio})_A F(\lambda_{em},\lambda_{ex}^A) + \alpha F^D(\lambda_{em},\lambda_{ex}^D)$$

where $(\text{ratio})_A$ and α are the two weighting factors for the two components. Therefore,

$$(\text{ratio})_A = \frac{F(\lambda_{em},\lambda_{ex}^D) - \alpha F^D(\lambda_{em},\lambda_{ex}^D)}{F(\lambda_{em},\lambda_{ex}^A)} = \frac{\varepsilon^A(\lambda_{ex}^D)}{\varepsilon^A(\lambda_{ex}^A)} + d^+E\frac{\varepsilon^D(\lambda_{ex}^D)}{\varepsilon^A(\lambda_{ex}^A)}$$

where ε is the extinction coefficient of the fluorophore and d^+ is the fraction of DNA labeled with donor. When donor labeling efficiency is 100%, d^+ equals one. E can then be calculated from the above equation. Here, λ_{ex}^D was 513 nm and λ_{ex}^A was 648 nm.

The E_{FRET} values were plotted against metal ion concentration [M] using the software OriginPro 8 and fit to a model with one metal ion bound to the DNAzyme, in order to determine the apparent dissociation constant (K_d) for folding.[48]

$$E_{FRET} = E_{FRET(0)} + \frac{C[M]}{K_d + [M]}$$

CD experiments. To perform the CD studies, a noncleavable substrate analogue, called 17DS, was used where the single ribo-A of 17S was replaced by a deoxy-A (Figure 1a). 17E and 17DS (200 μM each) were annealed in 50 mM Na-HEPES acetate buffer (pH 7.5) by heating the sample at 85 °C for 5 min and subsequently cooling it to room temperature over 1.5 h and then to 4 °C for ∼1−2 h. CD spectra were recorded on a JASCO J-515 spectropolarimeter in 1 mm path length cuvette using 500 μL sample volume. Each spectrum was recorded over a wavelength range of 340−200 nm using a scan speed of 50 nm/s and 0.5 s response time. The final concentration of DNA was diluted to 40 μM for all the experiments. By using a short path length cuvette and a high DNA concentration, buffer absorbance due to Na-HEPES was minimal. (A blank spectrum recorded with Na-HEPES buffer showed minimal noise in the 240−220 nm region under these conditions, whereas if a 1 cm cuvette with 4 μM DNA was used, significant noise was seen at that region.)

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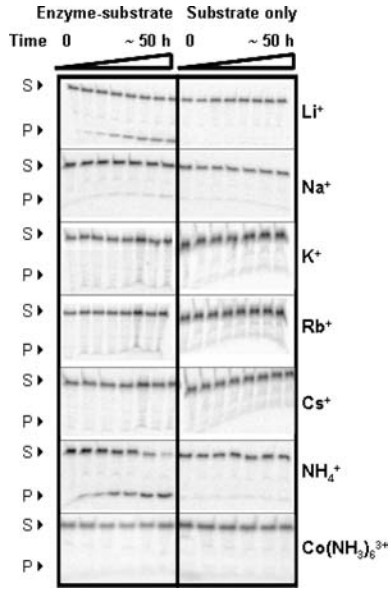

Figure 2. Representative gels for the 8-17 DNAzyme reaction in the presence of 4 M monovalent ions (Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+) and 80 mM $Co(NH_3)_6^{3+}$ in 50 mM Na-HEPES, 25 mM EDTA, pH 7.5. The left panels are reactions carried out in the presence of both enzyme and substrate, and the right panels are the control reactions carried out in the absence of enzyme. S and P indicate the position of uncleaved substrate and product bands respectively.

Divalent metal ions were titrated by incremental addition of concentrated metal solution, such that the total volume increased by <5%. In the case of monovalent ion titrations, a large volume of stock monovalent solution (5 M) had to be added, and therefore the DNA concentration was kept constant by adding in concentrated DNA stock upon metal addition.

To monitor the formation of Z-DNA, the CD signal at 294 nm (CD_{294}) was plotted against metal concentration [M] using Origin software and fit to the following equation to obtain K_d from CD data. Since the value of the CD signal changed from positive to negative with change in metal concentration, a constant was added to all the data points before curve fitting (see Supporting Information, S4).

$$CD_{294} = \frac{C[M]}{K_d + [M]}$$

Results

Activity of the 8-17 DNAzyme in the Presence of Monovalent Ions and $[Co(NH_3)_6]^{3+}$. The activity of the 8-17 DNAzyme was measured in the presence of alkali metal ions (Li^+, Na^+, K^+, Rb^+, and Cs^+) and NH_4^+, each at 4 M concentration. All the assays were carried out in 50 mM HEPES-acetate buffer, pH 7.5 which contained 25 mM EDTA. Our results showed that if EDTA was not present in the buffer, trace amount of divalent metal contaminants present in the buffer caused significant cleavage after ∼2 h, and thus, it was essential to include EDTA in the buffer.

Figure 2 depicts representative gels for the activity assays performed using 4 M monovalent ions and 80 mM $[Co(NH_3)_6]^{3+}$. The total time for which the reaction was carried out varied between 45−55 h. The left panels show the substrate cleavage in the presence of DNAzyme (called enzymatic cleavage), while the right panels display substrate cleavage in the absence of the DNAzyme (called background cleavage) under identical conditions. In the presence of Li^+ and NH_4^+, the intensity of product band can be seen increasing with time

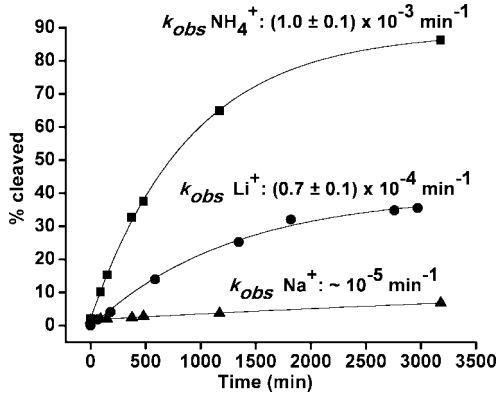

Figure 3. Kinetic trace for the 8-17 DNAzyme reaction in the presence of 4 M metal chloride solutions in 50 mM Na-HEPES and 25 mM EDTA (pH 7.5). The observed rate constants (k_{obs}) that are reported are an average of three independent experiments.

when DNAzyme is present, suggesting the 8-17 DNAzyme is active in these two monovalent ions. A small amount of cleavage was also observed for Na^+ (<10% in 50 h) over the background cleavage. In the case of the other monovalent ions (K^+, Rb^+, and Cs^+), no significant difference was observed between enzymatic cleavage and background cleavage after ~50 h. The percent cleavage is plotted vs time in Figure 3. The observed rate constant (k_{obs}) for 4 M Li^+ and NH_4^+ are 7×10^{-4} and 1×10^{-3} min^{-1}, respectively (calculated as an average of three independent trials). This rate constant is at least 4−5 orders of magnitude lower than the estimated rate constants at pH 7.5 with the most effective divalent metal cofactors, 100 μM Pb^{2+} (estimated $k_{obs} \sim 220$ min^{-1}) and 10 mM Zn^{2+} (estimated $k_{obs} \sim 50$ min^{-1}) and ~3 orders of magnitude lower than with 10 mM Mg^{2+} ($k_{obs} \sim 1$ min^{-1}).[62] However, the rate constant with monovalent ions still represents a rate enhancement of $\sim 10^7$ over the spontaneous rate of cleavage for the dinucleotide junction A−rA.[46]

In addition to monovalent ions, the exchange inert complex $[Co(NH_3)_6]^{3+}$ has also been used to probe if direct metal coordination is required for catalysis in ribozymes.[37,39,42] We tested the activity of the 8-17 DNAzyme in the presence of 80 mM $[Co(NH_3)_6]^{3+}$, and did not observe any noticeable rate enhancement in enzymatic cleavage over background cleavage (Figure 2, bottom panels).

Folding Studies Using FRET. Metal ion-dependent folding of the 8-17 DNAzyme was probed by FRET which can provide information on the relative distance between the arms of the DNAzyme when labeled with fluorescent donor/acceptor pair (Figure 1b). In order to carry out the FRET studies, a noncleavable analogue of the substrate, where the single RNA base was replaced by a DNA base, was used. Additionally, the DNAzyme complex was extended by two base pairs on both ends to increase the stability of the complex for FRET studies. The enzyme was labeled on the 5′ end with the acceptor fluorophore, Cy5, and the substrate was labeled on the 5′ end with the donor fluorophore, Cy3.

Figure 4a shows the typical FRET spectra for monovalent ions. The samples were excited at 513 nm, and emission spectra were collected from 530 to 700 nm. There are two main emission peaks: the peak at ~562 nm is from the donor (Cy3), and the acceptor (Cy5) peak at ~663 nm can be attributed to energy transfer from the donor. As the concentration of the metal ion was increased, there was a decrease in the intensity of the donor peak and an increase in the intensity of the acceptor peak. A considerable dilution effect on the fluorescence signal was observed due to the large volume increase upon addition of a large amount of monovalent ion solution, and thus anticorrelated fluorescence intensity changes of the donor and acceptor were not observed directly. However, the use of the $(\text{ratio})_A$ method[76] took into account the dilution effect and E_{FRET} increase was seen as the concentrations of the ions was increased. E_{FRET} changes in the presence of monovalent metal ions and NH_4^+ were obtained as a function of their concentrations (Figure 4b). Interestingly, in contrast to activity assays, where activity was observed only in the presence of Li^+, NH_4^+ and, to a lesser extent with Na^+; folding was seen with all the monovalent ions investigated. The K_d values obtained can be placed in two groups: stronger K_d for Li^+, Na^+ and NH_4^+ induced folding (0.228, 0.212 and 0.218 M respectively) and weaker K_d for Rb^+ and Cs^+ induced folding (0.359 and 0.344 M respectively).

Since a high concentration of monovalent metal ions was used for the FRET studies, a control experiment was performed in order to exclude artifacts associated with direct metal ion/fluorophore interactions. The fluorescence spectra of Cy3 (donor)-only labeled 17E-Cy3-17S complex showed no spectral shift upon titrating the monovalent ions into the DNA solution and the decrease in the intensity of the Cy3 (563 nm) peak was due to the dilution effect (see Supporting Information, S2). The quenching effects at high concentrations of the metal ions were insignificant (<5% quenching by 2.5 M Li^+, Na^+, Cs^+, NH_4^+ and 18% quenching by 2.5 M Rb^+).

For comparison to the folding in the presence of monovalent ions, divalent metal ion-dependent folding was also studied with selected transition and alkaline earth metal ions. Figure 5a shows the plot of E_{FRET} values vs metal concentration for Zn^{2+} and Cd^{2+}. The folding in the presence of these transition metal ions requires much lower concentration of metal ions, with K_d values of 3.10 and 3.06 μM for Zn^{2+} and Cd^{2+}, respectively. The alkaline earth metal ion-dependent E_{FRET} showed two distinct kinds of folding: one in the presence of Mg^{2+} and Ca^{2+} which induced higher saturated E_{FRET} states ($E_{FRET} \sim 0.66$) accompanied by stronger K_d values of 0.717 and 0.700 mM. respectively, as compared to Sr^{2+} and Ba^{2+} ($E_{FRET} \sim 0.58$) with K_d values of 1.09 and 1.06 mM respectively (Figure 5b). In general, the K_d values obtained for all the divalent metal ions studied are much stronger than those obtained with monovalent metal ions, indicating that a higher concentration of monovalent ions is required for folding of the DNAzyme. It should be noted that although Pb^{2+} is the most efficient cofactor for the 8-17 DNAzyme, previous FRET studies have shown that global folding is not observed in the presence of Pb^{2+}.[71,72]

Structural Changes Using CD. We were interested in CD studies because previous reports have shown that at high salt concentration, certain DNA sequences (particularly those containing alternate GC bases) can change from the right handed B-form helix to the left handed Z-form helix, and this transition can be monitored by CD.[78−87]

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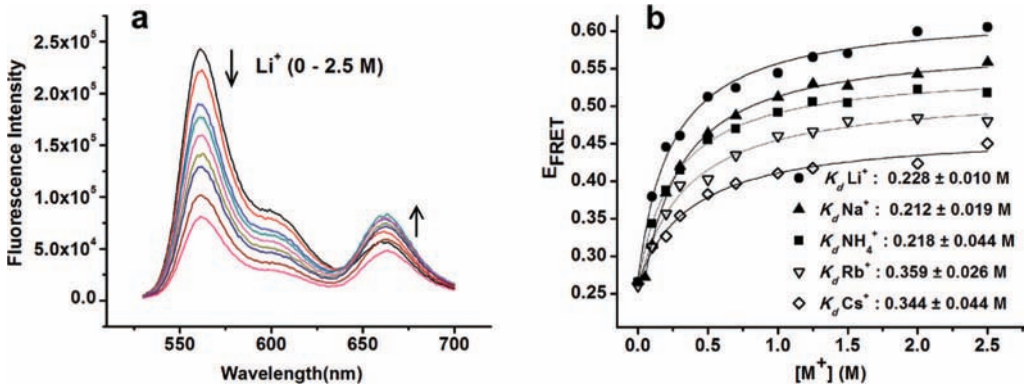

Figure 4. (a) Typical fluorescence spectra of the 8-17 DNAzyme construct used to probe FRET in the presence of monovalent ions. (b) Monovalent ion-dependent E_{FRET} changes in the 8−17 DNAzyme. The K_d values reported are an average of two independent measurements.

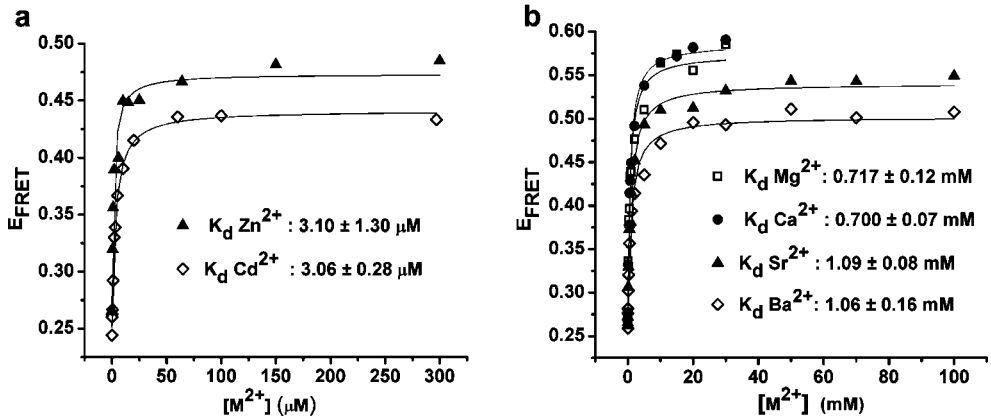

Figure 5. Divalent metal ion-dependent E_{FRET} changes in the 8-17 DNAzyme. Dependence of E_{FRET} on the concentration of (a) transition metal ions (b) alkaline earth metal ions. The K_d values reported are an average of two independent measurements. Note: The E_{FRET} value does not change in the presence of increasing concentrations of Pb^{2+}, which is the most effective cofactor for this DNAzyme (see references 71, 72).

Figure 6a is a depiction of typical CD spectra of B-DNA (red line) characterized by a negative signal at ~255 nm and positive signal at ~277 nm, and Z-DNA (blue line) characterized by a positive signal at ~272 nm and a negative signal at ~294 nm. Previous reports have shown that complete B→Z transitions occurred in DNA containing alternating GC bases at ~2.5 M Na^+. The CD spectra of the DNAzyme complex was recorded using increasing concentrations of monovalent ions Li^+, Na^+, Rb^+, Cs^+, NH_4^+ (Figure 6, b−f, respectively). Remarkably, as the concentration of monovalent ion was increased from 0−2 M, a small negative peak of increasing intensity was seen growing in at ~294 nm, which corresponds to the position of the signature negative peak reported for Z-DNA. Further increase in intensity of this negative peak was not observed upon increasing the ion concentration. The intensity of the positive peak at ~275 nm decreased with the increase in monovalent ion concentration. Upon titration of Mg^{2+} (0−10 mM) and Zn^{2+} (0−300 μM), the same effect was observed (Figure 6, g and h, respectively). In contrast, when Pb^{2+} was added up to 380 μM, we did not observe any negative peak at this wavelength (Figure 6i). While we cannot rule out the possibility that Pb^{2+} may induce structural changes at concentrations higher than 380 μM, we did not investigate in the higher concentration range for the following reasons. First, Pb^{2+} precipitates in such a high concentration at pH 7.5; second, the 8−17 DNAzyme activity saturates at ~200 μM Pb^{2+};[62] therefore any findings beyond 200 μM Pb^{2+} will not correlate with enzyme activity; finally, the Pb^{2+} concentrations used for this CD study are in the same range as the Zn^{2+} concentrations. Since Zn^{2+} showed the most pronounced Z-DNA peak, the effect of Zn^{2+} and Pb^{2+} can be compared using similar concentrations.

Since the 8-17 DNAzyme does not contain a sequence that is known to form Z-DNA, it is expected that complete B→Z conversion does not take place. It is possible that a small fraction of the DNAzyme converts to the Z-form or that a particular tract in the DNAzyme sequence can promote the formation of Z-DNA. To test if the negative peak at 294 nm was typical of the 8-17 DNAzyme construct, we performed two control experiments. The DNA constructs used in the control experiments were titrated with Zn^{2+} as it had resulted in the most prominent Z-DNA peak in the 8-17 construct.

First, instead of the enzyme strand, a 24-mer DNA strand completely complementary to the substrate was used to form a DNA duplex (17DS/comp-17DS) and upon titration with Zn^{2+}, no spectral changes were observed (Figure 7a). The negative peak at 294 nm did not appear even at 340 μM Zn^{2+}, indicating that the enzyme strand is necessary for the formation of Z-DNA. Second, since Z-DNA formation is typically seen in regions of

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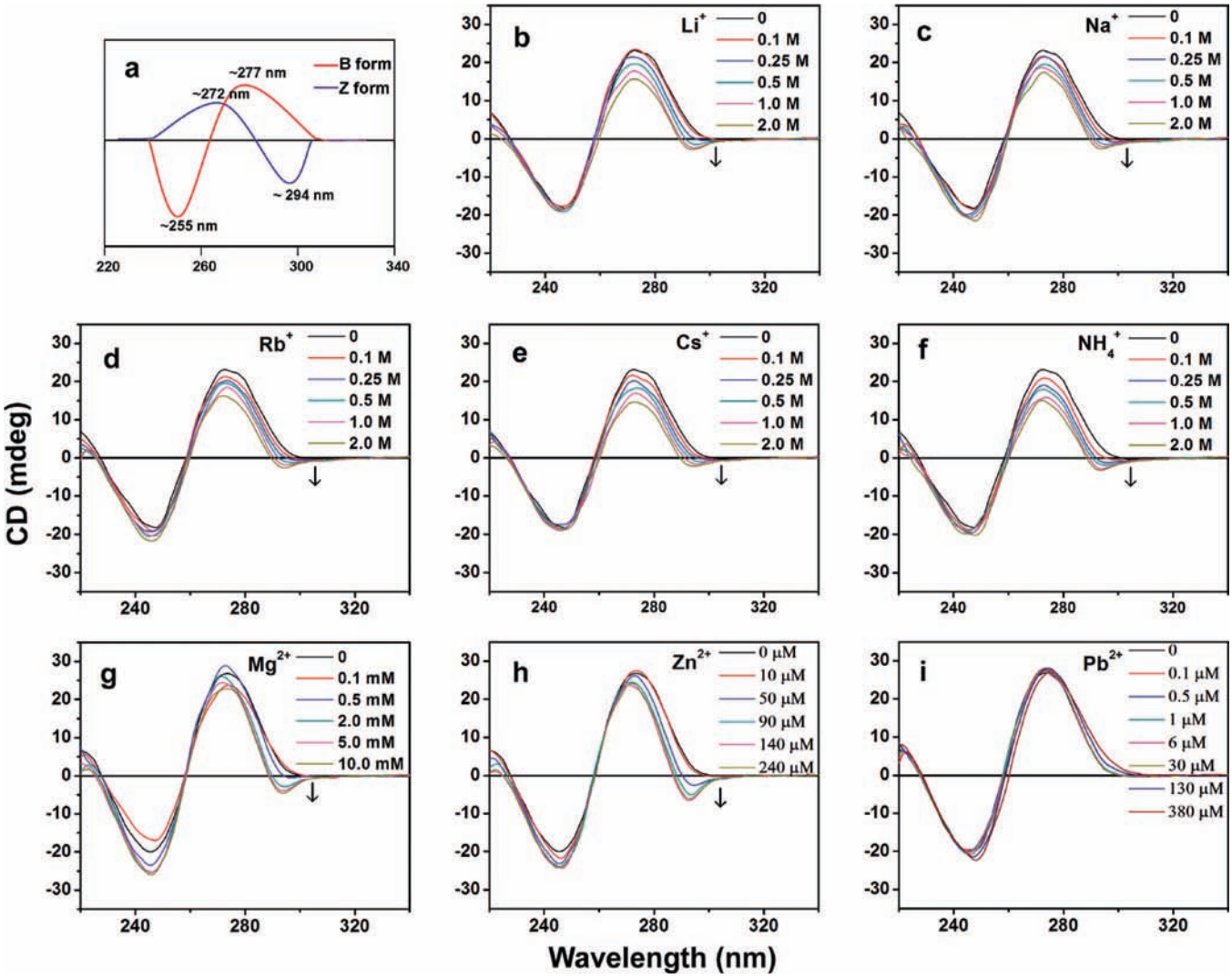

Figure 6. (a) Illustration of CD spectra for B-form (red line) and Z-form (blue line) DNA. (b−f) Monovalent ion- and (g−i) divalent metal ion-dependent structural changes in the 8-17 DNAzyme as observed by CD spectroscopy.

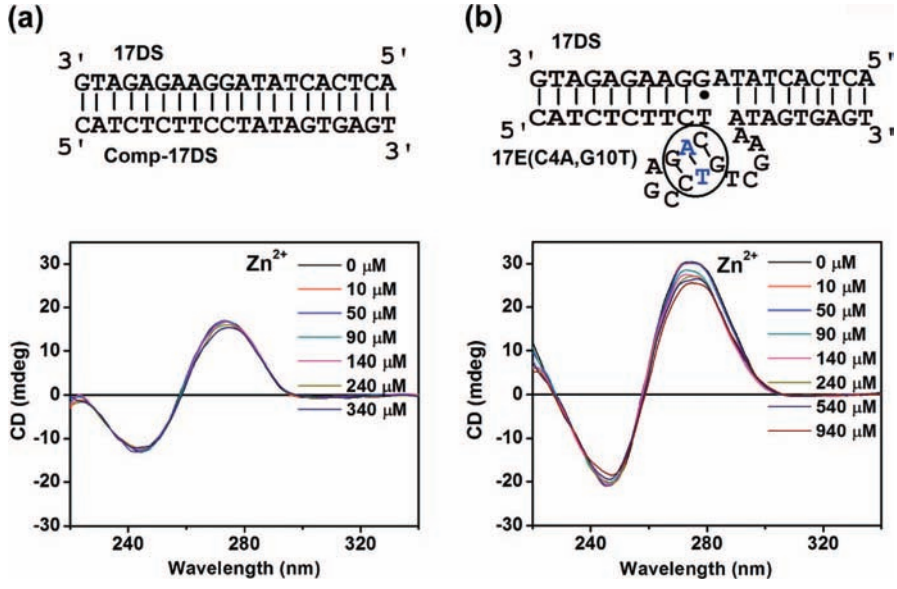

Figure 7. Structural changes in control constructs monitored by CD. Top: Sequence and predicted secondary structure, Bottom: CD spectra. (a) Duplex control (b) Control with a single base pair mutation in the catalytic core of the 17E enzyme. A GC base pair was mutated to an AT base pair and is indicated in blue color.

alternating GC, the three base pair stem on the enzyme which contains three GC base pairs was considered a potential region for Z-DNA formation. A single GC base pair was mutated to an AT base pair because AT base pairs have been known to inhibit Z-DNA formation,[88] and the CD spectra were recorded with Zn^{2+} (Figure 7b). Again, in this case the 294 nm peak was not observed, providing strong evidence that this region maybe contributing to the structural changes that we observe by CD in the 8-17 DNAzyme construct. Furthermore, enzymatic assays reported previously showed the GC to AT mutation resulted in a reduction in activity by a factor of ~5.5 in the presence of 5 mM Zn^{2+} in 50 mM Na-MES buffer (the k_{obs} went from 0.99 min^{-1} to 0.18 min^{-1}),[62] suggesting this mutation and associated Z- to B-DNA transformation may also play a role in enzymatic activity.

Finally, in order to exclude any artifacts from our instrumental or experimental conditions, a construct containing alternate GC base pairs that is known in literature to form Z-DNA at high Na^+ concentration[87] was used to perform CD studies on our instrument employing the same conditions as were used in the CD studies of the 8−17 construct (Supporting Information, Figure S3). Z-DNA formation was observed in this control construct. By using the intensity of the CD signal at 294 nm from the control construct as a standard and comparing the intensity of the 8−17 construct, we have estimated the percentage of Z-DNA formation in the 8-17 construct to be ~10% on a per mole, per nucleotide basis (see Supporting Information, Figure S3).

In order to quantify the metal dependence of the 8−17 construct, the CD signal at 294 nm was plotted against metal concentration for the monovalent ions (Figure 8a), Mg^{2+} (Figure 8b) and Zn^{2+} (Figure 8c). A clear transition from positive intensity to increasing intensity of a negative peak was observed. K_d (from CD) was determined to be 0.313, 0.235, 0.269, 0.236, 0.149 M for Li^+, Na^+, Rb^+, Cs^+, NH_4^+, respectively (See Supporting Information S4 for curve fittings). These K_d values from CD are in the similar range as the K_d of folding obtained from FRET studies (0.212−0.359 M for monovalent ions). The stronger K_d of the divalent metal ions (Mg^{2+}: 1.13 mM and Zn^{2+}: 55.6 μM) is also comparable to the K_d values obtained using FRET (Mg^{2+}: 0.71 mM and Zn^{2+}: 3.10 μM). These results indicate that metal-dependent global folding monitored using FRET is closely related to the structural changes that are examined using CD, suggesting that the formation of Z-DNA may play an important role for activity, except in the case of Pb^{2+}.

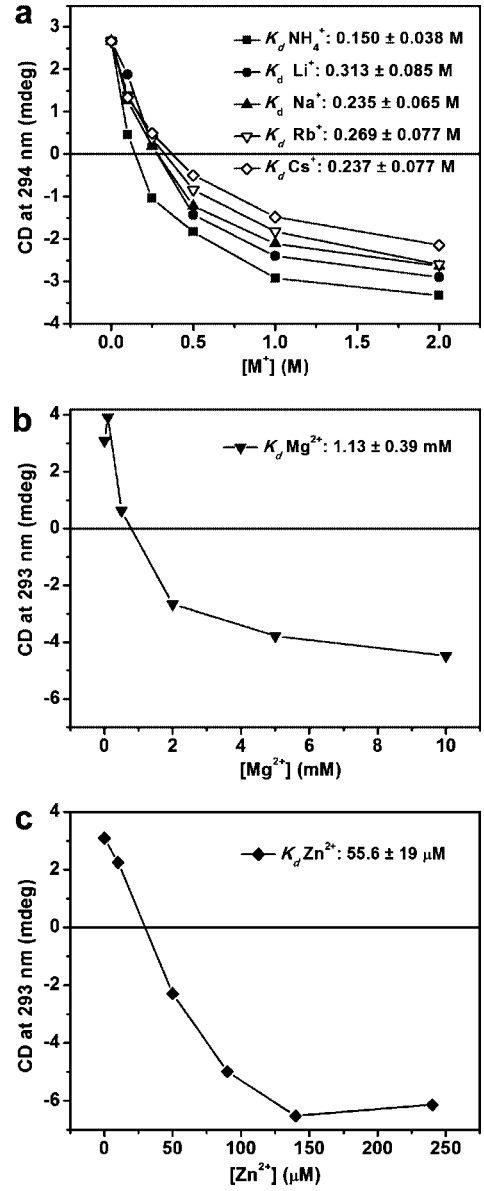

Figure 8. CD signal at 294 nm vs metal concentration for (a) monovalent ions (b) Mg^{2+} (c) Zn^{2+}. See Supporting Information S4 for curve fittings used for the determination of K_d values

Discussion

Comparison of Monovalent Ion-Dependent Activities between DNAzymes and Ribozymes. Previous work has shown that, while several ribozymes are active in the presence of divalent metal ions under physiological conditions, they are also active in the presence of very high concentration of monovalent cations.[39,41−43] Since the 8-17 DNAzyme shares similar secondary structure to the hammerhead ribozyme, such as the presence of a bulged three-way junction at the catalytic core, and ribonucleotide phosphodiester transfer activity, we compare the k_{obs} for the 8-17 DNAzyme in the presence of 4 M monovalent ions to that reported for the hammerhead ribozyme (Table 1). For the 8-17 DNAzyme, the k_{obs} obtained with the monovalent ions are much lower than those obtained for the hammerhead ribozyme. For example, for the hammerhead ribozyme, the 4 M Li^+-induced activity is only ~10-fold less than the 10 mM Mg^{2+}-induced activity, indicating that high concentrations of monovalent ion can replace divalent ions in catalysis. This result has led to the hypothesis that molar concentrations of monovalent ions can substitute millimolar concentrations of Mg^{2+} for catalysis in the hammerhead ribozyme, indicating primarily structural contributions of metal ions to catalysis.[10,39,41,42] In contrast, the 8-17 DNAzyme activity in the presence of 4 M monovalent ions (Li^+ and NH_4^+) is ~1000-fold lower than that in the presence of 10 mM Mg^{2+}, and ~200,000-fold slower than the estimated rate constant in the presence of 100 μM Pb^{2+}. Therefore, our studies indicate that the 8-17 DNAzyme, obtained by *in vitro* selections has evolved to have a more stringent requirement for divalent metal ions to obtain high activity as compared to the naturally occurring ribozymes. It also explains its excellent selectivity as a Pb^{2+} sensor over the large excess of monovalent metal ions.[63] Another major difference between

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Table 1. Comparison of Observed Rate Constants (k_{obs}) for the 8-17 DNAzyme, the Hammerhead Ribozyme and the G3 DNAzyme in the Presence of Selected Metal Ions and $Co(NH_3)_6^{3+}$ under Single Turn-Over Conditions

	k_{obs} (min^{-1})						
	Li^+	Na^+	NH_4^+	Mg^{2+}	Zn^{2+}	Pb^{2+}	$Co(NH_3)_6^{3+}$
8-17 DNAzyme	7.0×10^{-4} [a] (4 M)	$\sim 10^{-5}$ [a] (4 M)	1.0×10^{-3} [a] (4 M)	0.9[b] (10 mM)	1.3[d] (50.0[e]) (10 mM)	6.5[b] (220.0[c]) (100 μM)	N.D[a] (80 mM)
Hammerhead ribozyme	2.9×10^{-1} [f] (4 M)	7.5×10^{-3} [f] (4 M)	1.4×10^{-2} [f] (4 M)	2.2[f] (10 mM)	–	–	7.1×10^{-3} [f] (100 mM)
G3 DNAzyme	3.0×10^{-3} [g] (500 mM)	4.0×10^{-3} [g] (500 mM)	4.0×10^{-3} [g] (500 mM)	N.D[g] (20 mM)	–	–	N.D[g] (100 mM)

[a] Data from the present study and the reaction conditions were 50 mM HEPES and 25 mM EDTA (pH 7.5). [b] Data taken from ref 62 and the reaction conditions were 50 mM HEPES (pH 7.0) for Mg^{2+} assay and 50 mM MES (pH 6.0) for Pb^{2+} assay. [c] The Pb^{2+}-dependent cleavage was too fast to be measured at higher pH; however, from the pH-dependent rate profile it is estimated that the rate at pH 7.5 will be ~ 220 min^{-1}. [d] Data taken from ref 56, and the reaction conditions were 50 mM MES (pH 6.0). [e] The Zn^{2+}-dependent cleavage was too fast to be measured at higher pH; however, from the pH-dependent rate profile it is estimated that the rate at pH 7.5 will be ~ 50 min^{-1}. [f] Data taken from ref 42, and reaction conditions were 50 mM Tris (pH 8.0) for Mg^{2+}-dependent assay and 50 mM Tris and 25 mM EDTA (pH 8.0) for M^+ assays. [g] Data taken from ref 46, and reaction conditions were 50 mM HEPES and 1 mM EDTA (pH 7.0). N.D denotes not detectable, and – denotes not reported

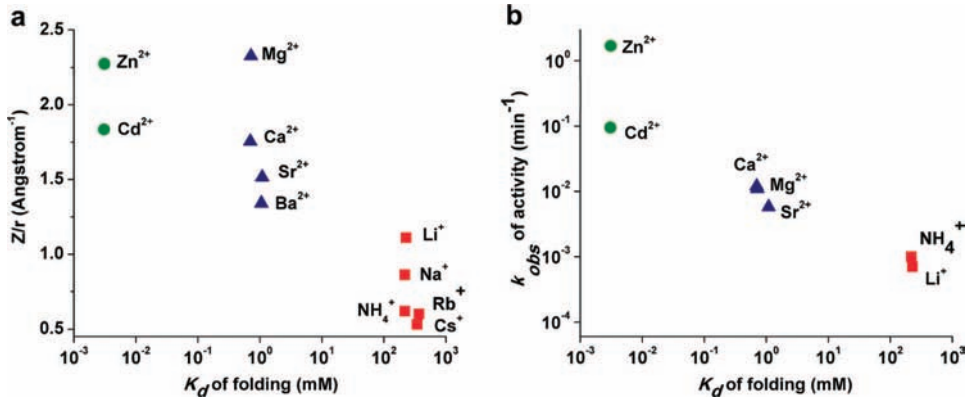

Figure 9. (a) Relationship between Z/r and K_d for monovalent and divalent ions (b) Relationship between k_{obs} and K_d for monovalent and divalent ions.

the two nucleic acid enzymes is the $[Co(NH_3)_6]^{3+}$-dependent activities. While $[Co(NH_3)_6]^{3+}$ alone can support hammerhead ribozyme activity, its role in 8-17 DNAzyme activity is not detectable, suggesting that outer-sphere metal coordination is not likely to operate, at least in the presence of $[Co(NH_3)_6]^{3+}$.

We also compared our results using the 8-17 DNAzyme to that of the G3 DNAzyme (Table 1) isolated by Geyer et al. using *in vitro* selection in the absence of any divalent metal cofactor.[46] In contrast to the hammerhead ribozyme and the 8-17 DNAzyme, the G3 DNAzyme displayed no activity in the presence of divalent metal ions and is a prototype monovalent ion-dependent DNAzyme. These DNAzymes utilize the monovalent metal ion more efficiently as they achieve similar k_{obs} ($\sim 10^{-3}$ min^{-1}) at a much lower concentration of 500 mM as opposed to 4 M concentration required by the 8-17 DNAzyme. These results indicate that laboratory selected DNAzymes may differ from naturally occurring ribozymes in their metal dependence because their active sites can evolve to have more stringent discrimination between different types of metal ions.

Correlation between Ionic Radii and Folding. The K_d values obtained by FRET for different metal ions were plotted as a function of Z (charge)/r (ionic radii) (Figure 9a). It can be seen that the monovalent ions which have the largest ionic radii, and thus the lowest charge density, have the weakest K_d (~ 100 mM), whereas the divalent transition metal ions have the highest Z/r ratio and the strongest K_d ($\sim 10^{-3}$ mM). It was previously reported that small ions are more effective than large ions in condensing DNA[89−92] and stabilizing the tertiary conformation of ribozymes.[93,94] Woodson and colleagues interpreted the greater stability of RNA with small metal ions in terms of electrostatic interactions only.[93,94] They discussed two factors for small, high charge density cations to be better in RNA folding. First, these can approach the RNA more closely, resulting in stronger attractive Coulombic interactions. Second, the excluded volume of each ion is smaller,[89,95] which lowers the entropic penalty for confining the ions to a small volume around the RNA. A similar observation in this study in relation to folding and ion sizes suggests that the observed folding is at least partially due to electrostatic interactions between ions and the DNAzyme.

Correlation between Activity and Folding. In order to examine a relation between folding and activity of the DNAzyme, we have coplotted the K_d of folding from FRET and k_{obs} from the cleavage reaction in Figure 9b. The activity of the 8-17 DNAzyme is dependent on metal cofactors with the following

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order of activity; $Pb^{2+} \gg Zn^{2+} \gg Cd^{2+} \gg Mg^{2+} \sim Ca^{2+} > Ba^{2+} \sim Sr^{2+} \gg \sim NH_4^+ \sim Li^+ \gg Na^+$. We found close correlation between folding and activity (stronger binding affinity in folding results in higher activity), except in the case of Pb^{2+} ion, which does not induce global folding of the two arms in the presence of 50 mM Na-HEPES, pH 7.[71,72] These correlations strongly suggest metal-ion dependent folding plays an important role in the catalytic function of DNAzymes.

CD Evidence for Z-DNA Formation. Since FRET experiments provide information only on global folding, but not on structural changes, we sought to use CD to search for any structural changes in the presence of different metal ions, and found very interesting evidence of a small percentage of Z-DNA formation, evidenced by the increase in ~294 nm peak with increasing concentration of metal ions. It is not surprising that the percentage of Z-DNA formation in the 8-17 DNAzyme is low because it contains only a small portion of alternate GC bases in the stem region that are known to form Z-DNA.[78−87] CD has previously been used to monitor structural changes in the hammerhead ribozyme,[96−99] HDV ribozyme,[100] hairpin ribozyme,[101] lead ribozyme,[102,103] RNA subunit of RNase P,[104,105] 10-23 DNAzyme,[106] Dk5 DNAzyme,[107] and a Ca^{2+}-dependent DNAzyme;[108] however to the best of our knowledge, this is the first study where metal dependent Z-DNA formation has been reported among DNAzymes and ribozymes. More importantly, since the K_d obtained from the CD studies are similar to those obtained from the FRET studies and activity assays, these results suggest that both metal ion-dependent folding and Z-DNA formation play important roles in monovalent ion- and divalent metal ion-dependent activities. The only exception is Pb^{2+}, in the presence of which, no global folding or Z-DNA formation was observed, indicating Pb^{2+}-dependent enzymatic function is based upon a complete different mechanism from those of monovalent metal ions and other divalent metal ions.

Conclusion

Monovalent ion-dependent activity of the 8-17 DNAzyme was examined and 4 M Li^+ and NH_4^+ showed ~1000-fold and ~200,000-fold lower activity compared to 10 mM Mg^{2+} and 100 μM Pb^{2+} respectively, indicating high selectivity of the DNAzyme for divalent metal ions. This is in contrast to the hammerhead ribozyme where the activity in 4 M Li^+ was only ~10-fold lower than that of Mg^{2+}. Interesting correlations between ionic radii of the metal ions, effective in folding and activity have also been observed, suggesting that, with the exception of Pb^{2+}, the most active metal ion, electrostatic interactions between metal ions and DNA are important in the folding and activity of the 8-17 DNAzyme. Structural changes monitored by CD, provide a very interesting evidence for Z-DNA formation in the presence of monovalent ions, Mg^{2+} and Zn^{2+}, but not in the case of Pb^{2+}, providing further proof that Pb^{2+} acts in a different manner as opposed to other active metal ions. To the best of our knowledge, this is the first report of Z-DNA formation in ribozymes and DNAzymes. Further investigation is required to gain insight into the sequence elements that maybe responsible for the formation of Z-DNA.

Acknowledgment. We thank Professor Martin Gruebele at the University of Illinois for the use of their CD instrument. We also thank Krishnarjun Sarkar, Apratim Dhar and Sharlene Denos for assistance with the CD measurements. This material is based upon work supported by the Office of Science (BER), U.S. Department of Energy, Grant No. DE-FG02-08ER64568, the U.S. National Science Foundation (CTS-0120978, DMR-0117792, and DMI-0328162), and the National Institutes of Health (ES016865).

Supporting Information Available: (1) Activity assay data for 8-17 DNAzyme starting with 2× and 5× enzyme−substrate solution; (2) fluorescence spectra to test direct interaction between fluorophore and metal ions; (3) CD spectra of control sequence; (4) curve fitting used to determine K_d for CD experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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