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Metal-Dependent Global Folding and Activity of the 8-17 DNAzyme Studied by Fluorescence Resonance Energy Transfer

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Abstract: The 8-17 DNAzyme is a DNA metalloenzyme catalyzing RNA transesterification in the presence of divalent metal ions, with activity following the order $Pb^{2+} \gg Zn^{2+} \gg Mg^{2+}$. Since the DNAzyme has been used as a metal ion sensor, its metal-induced global folding was studied by fluorescence resonance energy transfer (FRET) by labeling the three stems of the DNAzyme with the Cy3/Cy5 FRET pair two stems at a time in order to gain deeper insight into the role of different metal ions in its structure and function. FRET results indicated that, in the presence of Zn^{2+} and Mg^{2+}, the DNAzyme folds into a compact structure, stem III approaching a configuration defined by stems I and II without changing the angle between stems I and II. Correlations between metal-induced folding and activity were also studied. For Zn^{2+} and Mg^{2+}, the metal ion with higher affinity for the DNAzyme in global folding ($K_{d(Zn)} = 52.6\ \mu M$ and $K_{d(Mg)} = 1.36$ mM) also displays higher affinity in activity ($K_{d(Zn)} = 1.15$ mM and $K_{d(Mg)} = 53$ mM) under the same conditions. Global folding was saturated at much lower concentrations of Zn^{2+} and Mg^{2+} than the cleavage activities, indicating the global folding of the DNAzyme occurs before the cleavage activity for those metal ions. Surprisingly, no Pb^{2+}-dependent global folding was observed. These results suggest that for Pb^{2+} global folding of the DNAzyme may not be a necessary step in its function, which may contribute to the DNAzyme having the highest activity in the presence of Pb^{2+}.

Introduction

The interactions between metal ions and nucleic acids have been a long-standing focus of bioinorganic chemists because advances in this area can have significant impact in many other fields including biotechnology and medicine.[1−11] A primary example is the study of binding of *cis*-platinum and its analogues with DNA, whose information allows the design of more effective anticancer drugs.[1,4,6,7,10] Of particular interest are recent discoveries that DNA and RNA can catalyze a number of biological reactions like protein enzymes, and these catalytic nucleic acids usually require metal ions for function.[8,12−17] Therefore these catalytic nucleic acids (also called deoxyribozyme/ribozyme or DNA/RNAzymes) have become new members of metalloenzymes, and elucidation of the roles metal ions in the structure and function of DNA/RNAzymes has been a new focus of research for bioinorganic chemists. Toward this goal, a number of biochemical and biophysical studies have been devoted to study the role of metal ions in folding and catalytic activity of ribozymes. For example, fluorescence resonance energy transfer (FRET)[18,19] and electron paramagnetic resonance (EPR)[20] have been used to study metal-dependent ribozyme conformation changes; spectroscopy titrations have been used to study ribozyme/metal coordination chemistry,[21−23] and mechanistic studies on the role of metal ions in ribozyme catalysis have been extensively carried out.[17,24]

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In comparison to the considerable amount of work performed to understand the role of metal ions in ribozyme catalysis, the interactions between metal ions and DNAzymes are much less studied. Unlike ribozymes, no naturally occurring DNAzymes have been isolated in Nature and all DNAzymes have been obtained in the laboratory through a combinatorial process called *in vitro* selection.[16,25−29] Since their discovery[25] in 1994 many DNAzymes that can catalyze a broad range of chemical and biological reactions have been isolated.[16,26−30] In comparison with protein- or RNA-based enzymes, DNAzymes are less susceptible to hydrolysis and are more cost-effective to produce, and thus have many potential applications as antiviral agents,[31] biosensors for metal ions[32−35] and organic molecules,[36,37] components for DNA-based logic gates,[38] components for nanomotors,[39,40] and proofreading units in nanomaterial assembly.[41] To keep up with the significant advances in the application front, a fundamental understanding of DNAzymes, especially DNAzyme/metal interactions, would be crucial,[30,42−45] because divalent metal ions are required for most DNAzyme activity.

Among the many DNAzymes, we are especially interested in studying RNA-cleaving DNAzymes such as the 8-17 DNAzyme,[31,45−48] since it has been used the most extensively in the applications mentioned above. Interestingly, the same catalytic motif has been selected by several different research groups under different selection conditions, such as in the presence of 10 mM Mg^{2+},[31] 0.5 mM Mg^{2+}/50 mM histidine,[46] or 0.1 mM Zn^{2+}.[47] Surprisingly, DNAzyme activity is the highest in the presence of Pb^{2+}, with the following order: $Pb^{2+} \gg Zn^{2+} \gg Mg^{2+}$.[30,44,45] As a first step in understanding the role of these metal ions in the structure and function of the DNAzymes, we focus our study on metal ion-dependent folding of the DNAzyme and test whether major conformational changes are required to activate the DNAzyme. Toward this goal, we chose to use FRET to carry out the folding study, because FRET has been widely applied to study metal-dependent DNA/RNA folding.[49] Herein, we report the global folding of the 8-17 DNAzyme induced by Zn^{2+}, Mg^{2+}, and Pb^{2+} and its relation to the activity of the DNAzyme. A surprising and significant finding is that, unlike other metal ions investigated, the Pb^{2+} ion does not promote global folding, and this behavior may explain its high activity.

Materials and Methods

Oligonucleotides. All oligonucleotides except internal Cy5-labeled ones were purchased from Integrated DNA Technologies Inc. and were purified by HPLC. Internal Cy5-labeled DNAs were prepared by reacting DNA containing internal amine-modified T base (purchased from Integrated DNA Technologies Inc.) with NHS-ester-modified Cy5 (GE Healthcare, PA) and purified by HPLC after ethanol precipitation. DNA sequences and the corresponding nomenclature used in this study are shown in Figure 1.

Screening 8-17 Variants' Activity. The 8-17 variants listed in Figure 2 were tested for their activities in the presence of 20 μM Pb^{2+}. The cleavage activity assays were performed in the presence of 5000-fold excess enzyme strand under single-turnover conditions. The enzyme and the radioisotope-labeled cleavable substrate, 17S, were annealed in 50 mM Na-MES (pH 6.0) buffer by heating at 85 °C for 5 min and cooling to room temperature over 30 min. The reaction was initiated by adding the same volume of Pb^{2+} solution to the annealed enzyme−substrate solution. The final concentrations were 5 μM 17E DNAzyme, 1 nM 17S substrate, and 20 μM Pb^{2+}. The reaction was quenched at various time points by adding aliquots (usually 4 μL) of reaction mixture into a stop-buffer containing 8 M urea, 50 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. The reaction products and uncleaved substrate were separated by electrophoresis on a 20% denaturing polyacrylamide gel and analyzed with a PhosphorImager.

17E(sda5) Cleavage Activity Assays. The Cy3-labeled cleavable substrate (Cy3-17S) was used for monitoring the cleavage reaction. The sample was prepared by annealing 5 μM enzyme with 4 μM substrate in 50 mM Na-HEPES acetate (pH 7.0) at 85 °C for 5 min and subsequently cooling it to 4 °C over 2 h. HEPES buffer was chosen over Tris, since the activity of the DNAzyme was in general lower in Tris buffer probably due to better metal-chelating ability.[50,51] Reaction was started by mixing 10 times concentrated metal ions into the DNAzyme complex. The reaction was quenched at various time points by adding aliquots (usually 10 μL) of reaction mixture into stop-buffer containing 8 M urea, 50 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. The reaction products and uncleaved substrate were separated by electrophoresis on 20% denaturing polyacrylamide gels and analyzed with a fluorescence imager (FLA-3000, Fujifilm). The percentage of product was plotted against time. The observed rate constant (k_{obs}) was obtained by nonlinear curve fitting using the software SigmaPlot based on the equation $y = y_0 + a(1 - e^{-kt})$, where y is the percentage of product at time t, y_0 is the background cleavage at $t = 0$, a is the fraction reacted at $t = \infty$, and k is the observed rate constant, k_{obs}.

FRET Sample Preparation and Measurement. To study metal-induced folding, 10 μM DNAzyme strand and 10 μM substrate strand were annealed in 50 mM Na-HEPES acetate buffer (pH 7.0) by heating the sample solution at 85 °C for 5 min and subsequently cooling it to 4 °C over 2 h. To maximize hybridization for FRET studies, the annealed product was purified with a 16% native polyacrylamide gel at 4 °C using a procedure described previously.[43,52] All the steps were carried out at 4 °C to minimize dehybridization. The purified sample was dissolved in 50 mM Na-HEPES acetate, pH 7.0, and its concentration was determined by measuring its absorption at 260 nm. The sample was then diluted to <100 nM using the same buffer; at such a low concentration, the fluorophore concentrations were diluted enough to

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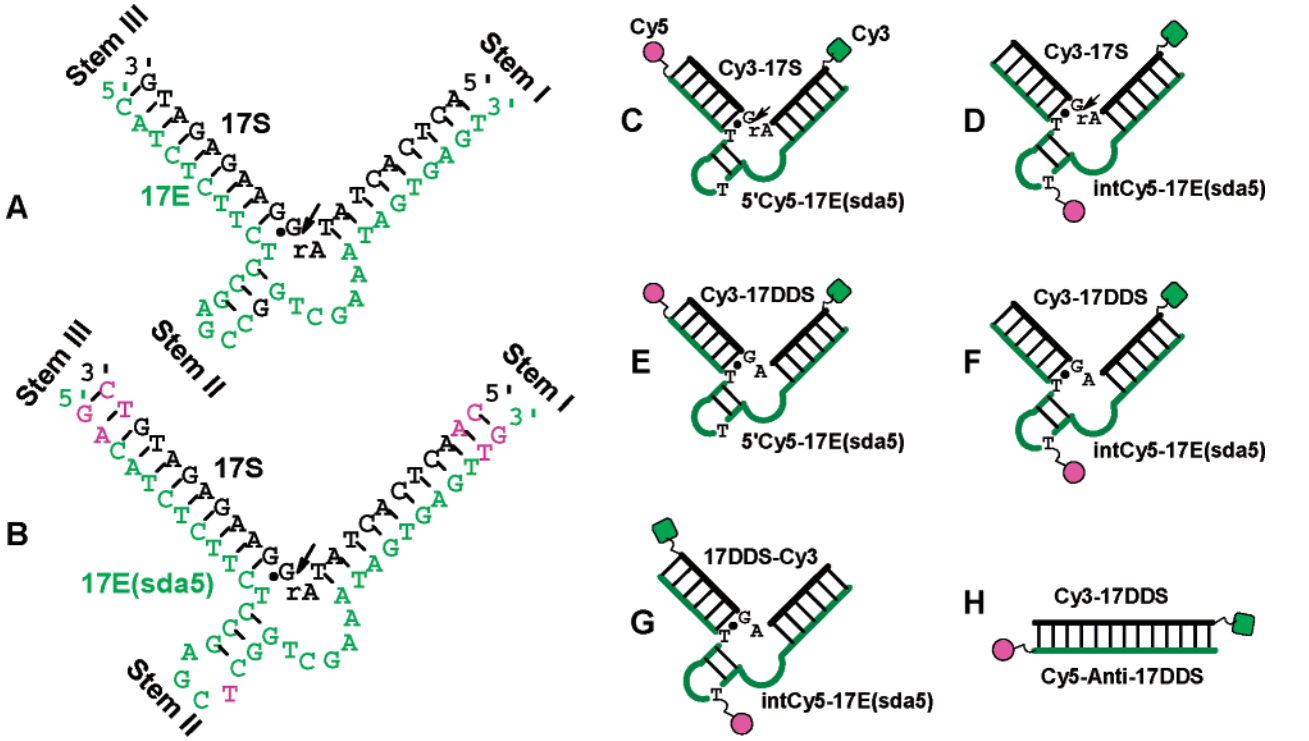

Figure 1. (A) Secondary structure of the 8-17 DNAzyme. (B) Secondary structure of the 17E(sda5)-17S. (C) Construct for enzyme assay: cleavable Cy3-labeled substrate (Cy3-17S) hybridized with Cy5-labeled enzyme (5′Cy5-17E(sda5)). (D) Construct for enzyme assay: cleavable Cy3-labeled substrate (Cy3-17S) hybridized with internal Cy5-labeled enzyme (intCy5-17E(sda5)). (E) Construct for probing FRET between stems I/III: noncleavable Cy3-labeled all-DNA substrate analogue (Cy3-17DDS) hybridized with 5′Cy5-17E(sda5). (F) Construct for probing FRET between stems I/II: Cy3-17DDS hybridized with intCy5-17E(sda5). (G) Construct for probing FRET between stems II/III: noncleavable substrate labeled with Cy3 at the end of 3′(17DDS-Cy3) hybridized with intCy5-17E(sda5). (H) Construct for a duplex control with Anti-17DDS.

avoid inner-filter effects.[49] Concentrated divalent metal ions were added to the sample to initiate FRET studies.

Steady-state fluorescence emission spectra were recorded on a Fluromax-2 fluorometer (HORIBA Jobin Yvon Inc., Edison, NJ). To avoid polarization artifacts, the polarizer was set under "magic angle conditions".[53] Fluorescence measurements were carried out at 10 °C to minimize dehybridization of the DNAzyme−substrate complex. The FRET efficiency (E_{FRET}) was calculated using the $(\text{ratio})_A$ method.[43,49] Data analysis was performed by using Microcal Origin 6.0.

Calculation of FRET Efficiencies Using the $(\text{ratio})_A$ Method. A detailed description of the $(\text{ratio})_A$ method can be found in the literature.[49] For a FRET pair, when the donor (D) is excited, the nearby (10−100 Å) acceptor (A) can receive energy from the donor with efficiency E as expressed as[49]

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

where R is the donor-to-acceptor distance. R_0 is defined by $R_0 = 8.785 \times 10^{23} \times \Phi^D \times \kappa^2 \times \eta^{-4} \times J(\nu)^6$ 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.[54] η is the refractive index of the media. $J(\nu)$ is the overlap integral of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor.

In the $(\text{ratio})_A$ 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 $F(\lambda_{em}, \lambda_{ex}^A)$ excited at λ_{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,

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

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 1. E can then be calculated from the above equation. Here, λ_{ex}^D was 513 nm and λ_{ex}^D was 648 nm.

Results

The 8-17 DNAzyme and Its Variants Used in This Study. The secondary structure of the *trans*-cleaving 8-17 DNAzyme is shown in Figure 1A. The enzyme strand (called 17E) consists of an all-DNA oligonucleotide, while the substrate strand (called 17S) is a DNA/RNA chimera containing a single ribo-adenosine (rA) that serves as the cleavage site (indicated by an arrow). The 17E recognizes the 17S by Watson−Crick base pairing to form a bulged three-way junction structure with three stems (I, II and III). In the presence of a metal cofactor, the enzyme catalyzes hydrolytic cleavage of the substrate.[31,46,47] In order to probe metal-dependent folding of this DNAzyme, it is necessary to place a FRET pair such as Cy3/Cy5 not only between stems I and III (Figure 1E) but also between stems I and II (Figure 1F) and between stems II and III (Figure 1G). More importantly,

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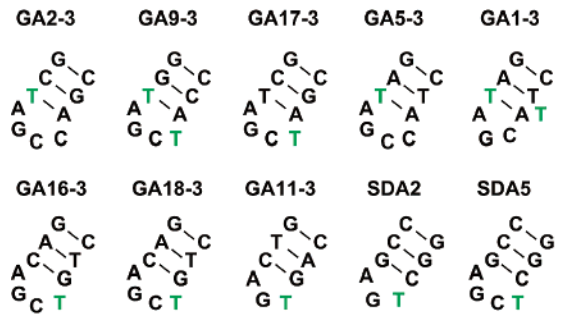
A



B

8-17 Variants	Cleaved Fraction at 5 min	Cleaved Fraction at 10 min
17E	0.9	0.9
GA2-3	0.2	0.3
GA9-3	0.01	0.02
GA17-3	0.2	0.3
GA1-3	0.1	0.2
GA5-3	0.03	0.06
GA16-3	0.3	0.5
GA18-3	0.1	0.1
GA11-3	0.6	0.7
SDA2	0.7	0.8
SDA5	0.5	0.7

Figure 2. (A) Sequences and proposed secondary structures of 8-17 variants containing T base on the stem II for internal fluorophore labeling. Here, we show only the stem II region, and other parts of the variants are the same as 17E. (B) Activity of 8-17 variants by 20 μM Pb^{2+} in 50 mM Na-MES buffer (pH 6.0) under single-turnover conditions. A ^{32}P-labeled cleavable substrate 17S was used for all the variants.

to correlate metal-dependent folding with its activity, it is critical to ensure that placement of the fluorophores does not abolish metal-dependent enzymatic activity. It has been shown from previous studies that placing fluorophores at the 3′ or 5′ end of either 17E or 17S does not change the enzyme activity.[32] The placement of the fluorophore in the middle of 17E to probe stem II, however, is much trickier, as previous biochemical studies have shown that the stem loop sequence in the stem II is highly conserved.[30,44] To search for active 8-17 variants in which a fluorophore Cy5 can be attached to the middle of stem II (i.e., internal fluorophore labeling), we screened a number of 8-17 variants described by Li and co-workers[45] in three steps. In the first step, since internal fluorophore labeling is feasible through conjugation between an NHS-ester-modified fluorophore and internal dT base modified with an amine, 10 variants containing the T base around the end of stem II including the loop region (Figure 2A) were tested for the activity.

All the activity assays were carried out under single-turnover condition with the cleavable substrate 17S in the presence of 20 μM Pb^{2+} in 50 mM Na-MES buffer (pH 6.0). Out of the 10 variants tested, only three (GA11-3, SDA2, and SDA5) showed comparable activity to that of the original 17E construct as shown in Figure 1A (see Figure 2B). In the second step, two of the three variants, SDA2 and SDA5, were further tested for activity after the inserted T was replaced by an amine-modified T. Only SDA5 showed significant activity (~70% cleavage after 15 min reaction time) and, thus, was further conjugated to NHS-ester-modified Cy5 through the amine functional group in the third step. In this study, the SDA5 variant of the enzyme strand is called 17E(sda5) and its complex with the substrate strand 17S is called 17E(sda5)-17S. In addition to insertion of T for conjugation to a fluorophore, the enzyme and substrate strands were extended by two base pairs on each end of stems I and III to make more stable complexes under the experimental conditions. The melting temperature of the substrate/enzyme complex was measured to be 42 °C, indicating the complex is thermally stable at 10 °C, the temperature at which the FRET measurements were carried out.

Activity and Selectivity of 17E(sda5)-17S DNAzyme. Figure 3 shows the activity of 5′-Cy5-labeled and internal-Cy5-labeled 17E(sda5)-17S DNAzymes in the present of Pb^{2+}, Zn^{2+}, and Mg^{2+} ions. The experimental conditions for the activity assays were the same as those used for folding studies to ensure close correlation between the two studies. Constructs 1C and 1D with the cleavable substrate were used for 5′Cy5-17E(sda5) and intCy5-17E(sda5), respectively. Both DNAzymes showed the highest activity with Pb^{2+} ions followed by Zn^{2+} and then Mg^{2+} (Figure 3). The apparent K_d for Pb^{2+}, Zn^{2+}, and Mg^{2+} are <0.12, 1.15, and 53 mM, respectively. This result shows that the sda5 variant has the same metal selectivity as the 8-17 DNAzyme,[44] and thus the results obtained from studying the sda5 variant can be applied to the 8-17 DNAzyme. Similar activity was observed with the intCy5-labeled DNAzyme compared to 5′Cy5-labeled DNAzyme (Figure 3), indicating that the internal labeling of the DNAzyme with a bulky fluorophore does not significantly interfere with the activity, and thus the actual conformational changes of the stem loop can be probed by using the internal Cy5-labeled enzymes.

Folding Induced by Zn^{2+}. Metal-induced folding of the DNAzyme was studied using the constructs 1E, 1F, and 1G in Figure 1 for probing folding between stems I and III, I and II, and II and III, respectively. The samples were excited at 513 nm, and emission spectra were collected from 530 to 700 nm. Typical spectra show two main emission peaks (Figure 4). 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. In the absence of divalent metal ions, FRET efficiencies were measured to be 0.13, 0.21, and 0.19 on average for folding between stems I and III, I and II, and II and III, respectively. Metal titration experiments were performed for quantitative study of metal binding (Figure 5). Addition of Zn^{2+} to the constructs 1E (stems I, III) and 1G (stems II, III) caused a decrease in the 562 nm peak and an increase in the 663 nm peak (Figure 4), and thus the FRET efficiencies between stems I and III and stems II and III increased with increasing Zn^{2+} concentration (Figure 5A). However, no change was observed for the construct 1F (Figure 5A). Therefore, the results suggest that addition of Zn^{2+} induced stem III to approach the configuration constituting stems I and II to fold the DNAzyme into a more compact structure.

The FRET data for folding between stems I and III and between II and III were fit to a model with one Zn^{2+} ion bound to the DNAzyme, based on a ~1 ($n = 0.94$) Hill coefficient.[55] The fitting gave an apparent dissociation constant (K_d) of 52.6 ± 2.3 μM for folding between stems I and III and 83.2 ± 12.6 μM for folding between stems II and III.

To exclude artifacts associated with metal ion/fluorophore interactions in contributing to the observations, control experi-

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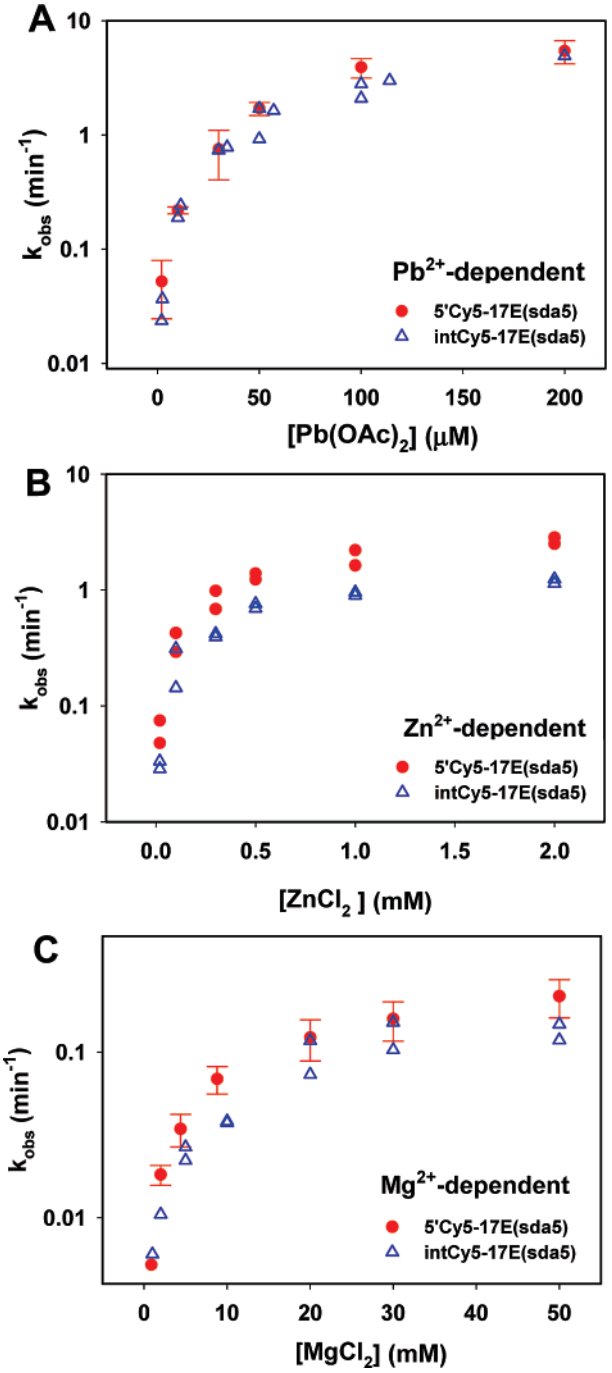

Figure 3. Single-turnover rate constants of 5′ Cy5-17E(sda5) (Figure 1C) and intCy5-17E(sda5) (Figure 1D) hybridized with Cy3-17S depending on (A) Pb^{2+}, (B) Zn^{2+}, and (C) Mg^{2+}. Reaction was carried out in 50 mM Na-HEPES (pH 7.0). All the experiments were repeated at least twice.

ments were performed. A control experiment with a Cy3 (donor)-only-labeled 17E(sda5)-Cy3-17S complex showed no spectral shape changes upon titirating the metal ions including Zn^{2+}, Mg^{2+}, and Pb^{2+} into the DNA solutions, and quenching effects by high concentration of those metal ions were insignificant (~5% quenching by 500 μM Zn^{2+} or 100 μM Pb^{2+} and ~12% quenching by 20 mM Mg^{2+}; see supplemental Figure S1). Another control experiment was performed with a 24-mer complete complementary DNA to the substrate strand (named Cy5-Anti-17DDS) to form double-stranded DNA with Cy3-17DDS (Figure 1H). Titration of Zn^{2+} into the sample did not

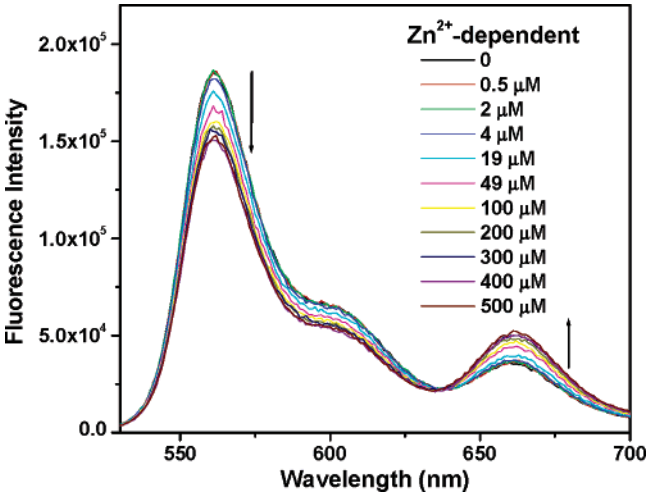

Figure 4. Typical fluorescence spectra of the construct 1E to probe FRET between stems I/III. The DNA sample was in 50 mM Na-HEPES (pH 7.0) and was excited at 513 nm. All the spectra were obtained at 10 °C.

change the duplex FRET efficiencies (supplemental Figure S2), which is consistent with the unbendable nature of the short DNA double helix. The FRET efficiency values in the Cy5-Anti-17DDS system were also much lower (~0.06) than those of the DNAzyme complexes (~0.12−0.25), suggesting that the two arms of the DNAzyme were not in a coaxial helix form. The control experiments helped support the observation that changes in FRET efficiencies were due to DNAzyme folding and not artifacts.

Folding Induced by Mg^{2+}. Mg^{2+} is a commonly used metal ion for *in vitro* selection of nucleic acid enzymes. For instance, the 8-17 DNAzyme has also been isolated in the presence of Mg^{2+} [31] and later shown to be more active with Ca^{2+}, Zn^{2+}, and Pb^{2+}.[44,46−48] The activity of the DNAzyme is much lower in the presence of Mg^{2+} ($k_{obs(2\ mM\ Mg2+)} = 0.018\ min^{-1}$, see Figure 3) than that in the presence of Zn^{2+} ($k_{obs(2\ mM\ Zn2+)} = 2.7\ min^{-1}$, see Figure 3). To compare folding induced by Mg^{2+} and Zn^{2+}, the DNAzyme was also titrated with Mg^{2+}, and the results are shown in Figure 5B. FRET efficiencies increased with increasing Mg^{2+} concentration for folding between stems I and III and between stems II and III with apparent K_d of 1.36 ± 0.24 and 0.840 ± 0.023 mM, respectively. No change in FRET efficiency was observed for folding between stems I and II, similar to that observed for Zn^{2+}-dependent FRET studies (Figure 5A). The metal-dependent folding affinity for Mg^{2+} (K_d = 1.36 mM) is much lower compared to Zn^{2+} (K_d = 52.6 μM).

Folding Induced by Pb^{2+}. Among all the metal ions tested, the DNAzyme showed the highest activity in the presence of Pb^{2+}, especially at low metal ion concentrations. Surprisingly, little changes in FRET efficiencies were observed for all three constructs even in the presence of 100 μM Pb^{2+} (Figure 5C), indicating no global folding occurs in the presence of Pb^{2+}, in contrast to those of Zn^{2+} and Mg^{2+}. To rule out artifacts associated with Pb^{2+}/fluorophore interactions, a control experiment was performed by titrating Pb^{2+} into the Cy3-17DDS/Cy5-Anti-17DDS duplex (supplemental Figure S2). The FRET efficiencies remained constant for this unbendable DNA, which confirmed that there was little Pb^{2+}/fluorophore interaction under experimental conditions (≤100 μM Pb^{2+}, HEPES buffer, pH

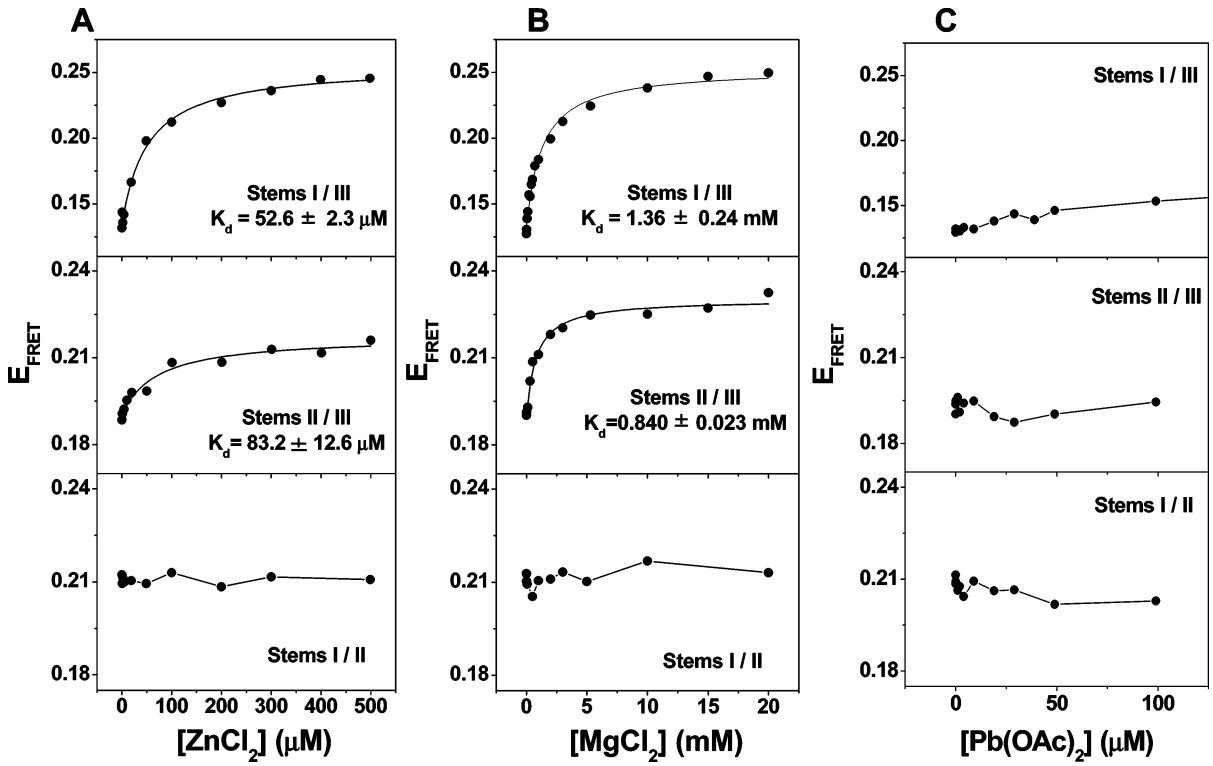

Figure 5. FRET efficiencies of the DNAzyme complex as a function of (A) Zn^{2+}, (B) Mg^{2+}, or (C) Pb^{2+}. Constructs 1E, 1F, and 1G were used for stems I/III, I/II, and II/III, respectively.

7.0). At higher concentrations, Pb^{2+} started to precipitate, and therefore, and no further data were acquired.

Discussion

Metal-Dependent Folding Based on the FRET Studies. The 8-17 DNAzyme contains three stems (Figure 1), similar to that in the hammerhead ribozyme.[56] FRET studies carried out in this work provide several new insights. First, little folding was observed between stems I and II upon addition any one of the three active divalent metal ions (Zn^{2+}, Mg^{2+}, and Pb^{2+}), indicating that the DNAzyme has a partially fixed configuration constituting stems I and II that is independent of these divalent metal ions. Second, both Zn^{2+} and Mg^{2+} induced folding between stems I and III, and between stems II and III, suggesting that stem III moves closer to the configuration defined by stems I and II in the presence of Zn^{2+} and Mg^{2+}. The difference between the two metal ions is their effects on folding; Zn^{2+} induced folding of stems I/III and stems II/III with K_d values of 52.6 ± 2.3 and 83.2 ± 12.6 μM, respectively, while Mg^{2+} induced folding of stems I/III and stems II/III with K_d = 0.840 ± 0.023 and 1.36 ± 0.24 mM, respectively. Finally, and perhaps the most surprisingly, no global folding was observed for any of the three stems in the presence of Pb^{2+}.

Correlation between Folding and Activity of the DNAzyme. In order to find out if the above metal-dependent global folding results based on FRET have any correlation with metal-dependent activity of the same construct, we carried out enzymatic assays under the same conditions. The observed activity rate constants k_{obs} and FRET efficiency E_{FRET} of stems I/III are plotted together in Figure 6. Several interesting findings emerge from such a comparison. First, complete folding of the DNAzyme was achieved by a lower concentration of Zn^{2+} than that of Mg^{2+}; this result is consistent with the fact that a lower concentration of Zn^{2+} is required than that of Mg^{2+} for activity. Second, in the presence of Zn^{2+} or Mg^{2+}, global folding was saturated at much lower concentrations than the cleavage activities, indicating that folding of the DNAzyme occurs before the cleavage activity for these two metal ions, and the DNAzyme requires more metal ions for further interactions with the DNAzyme to accomplish the cleavage reaction. The same result was also observed with the hammerhead ribozyme.[23,57−59]

Metal ions in nucleic acid enzymes have been categorized into four classes depending on their nature of interactions: nonspecific charge-screening metal ions neutralizing the negatively charged phosphate backbone of nucleic acids, electrostatically localized metal ions binding to well-defined, specific sites in nucleic acids with high affinity, inner-sphere site-bound metal ions interacting directly with nucleic acid ligands, and outer-sphere site-bound metal ions interacting through water molecules.[8,11,60,61] FRET used in this study is a low-resolution technique that may not be able to pinpoint the exact class of metal ions responsible for the observed global folding. For Mg^{2+} ions, high concentrations were required for folding. It is likely that Mg^{2+} ions act as both nonspecific electrostatic screening ions to neutralize backbone negative charges on the DNAzyme and site-specific metal ions facilitating cleavage activity. Folding studies of the hairpin ribozyme also showed that Mg^{2+} ions

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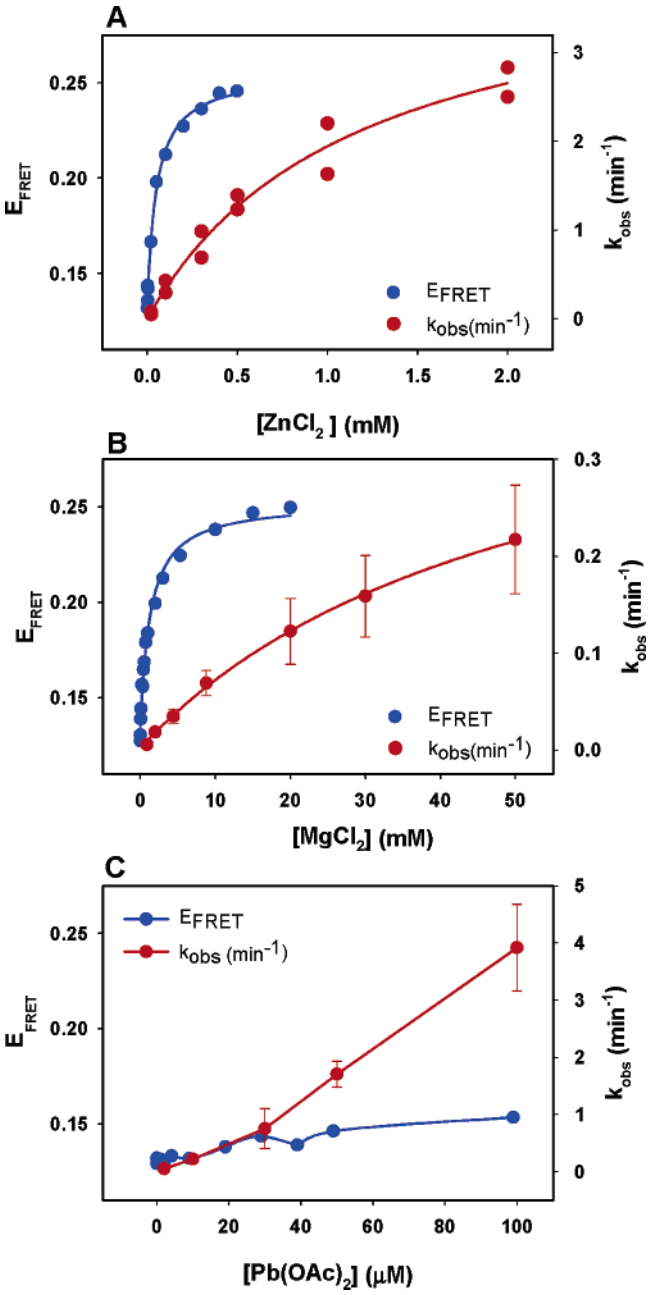

Figure 6. Comparisons of folding between stems I/III (from Figure 5) versus observed rate constants, k_{obs}, for the cleavage reaction of 5′Cy5-17E(sda5) enzyme (from Figure 3) in the presence of (A) Zn^{2+}, (B) Mg^{2+}, or (C) Pb^{2+}.

interact with the ribozyme both as initial diffuse binding and subsequent site-specific binding to form a final active conformation.[55]

Finally and more significantly, the DNAzyme lacked Pb^{2+}-induced global folding, even though it is the most active in the presence of Pb^{2+}. Since the concentrations of Pb^{2+} used in this investigation (0−100 μM) are much lower than those of Zn^{2+} (0−500 μM) and Mg^{2+} (0−20 mM) due to the Pb^{2+} precipitation problem at higher concentrations, we cannot rule out the possibility that the DNAzyme will fold at much higher Pb^{2+} concentrations (if one can solve the precipitation problem). However, a central feature of this investigation is correlation between global folding and enzyme activity in the same metal concentration range and under identical conditions. Under such conditions, little global folding was observed in the same metal concentration range as observed in enzyme activity. Even though global folding *might* be observed at much higher (e.g., millimolar) Pb^{2+} concentrations, those results would not have any correlation with enzymatic activity because the enzyme activity saturated at ~200 μM (see Figure 3A). Therefore Pb^{2+} is clearly different from Zn^{2+} and Mg^{2+}, as it could occupy a binding site different from those metal ions or bind to a similar site with a different mode so that no significant global conformational change occurs. Alternatively, it could be that the DNAzyme is prefolded into a Pb^{2+}-binding structure, and no further folding is needed for Pb^{2+}-induced catalysis. This could also explain the very fast reaction kinetics in the presence of Pb^{2+} compared to other divalent metal ions. These results suggest that for Pb^{2+}, global folding may not be a necessary step toward DNAzyme activity.

Conclusions

In conclusion, FRET measurements suggest that the 8-17 DNAzyme folds into compact structure(s) in the presence of Zn^{2+} or Mg^{2+} with stem III approaching a configuration constituting stems I and II without changing the angle between stems I and II. This global folding requires a lower concentration of Zn^{2+} or Mg^{2+} than those required for activity, suggesting that the DNAzyme requires more metal ions from the observed folded DNAzyme to an active enzyme. However, no conformational change was observed in the presence of Pb^{2+}, which is the most efficient cofactor for the DNAzyme activity. These results suggest that global folding may not always be a necessary step for the 8-17 DNAzyme catalytic activity. More spectroscopic and biological studies such as NMR and X-ray crystallography are required to investigate the nature of the interactions between the metal cofactors and the DNAzyme in detail.

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Supporting Information Available: Fluorescence spectra of donor only labeled enzyme−substrate complex and FRET measurements of a construct H in the presence of Zn^{2+}. This material is available free of charge via the Internet at http://pubs.acs.org.

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