In Vitro Selection of Hg (II) and As (V)-Dependent RNA-Cleaving DNAzymes

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ABSTRACT

DNAzymes (or catalytic DNA) are cell-free biomolecular recognition tools with target recognition sequences for charged molecules such as metal ions, antibiotics, and pharmaceuticals. In this study, using in vitro selection, large populations (e.g., \(10^{15}\)) of random DNA sequences were used as the raw material for the selection of “catalytic or functional molecules” for Hg\(^{2+}\) and As\(^{5+}\). From a random pool of 45-nt (Pool-A) and 35-nt (Pool-B) templates, we isolated RNA-cleaving catalytic Hg\(^{2+}\)- and As\(^{5+}\)-active DNAzymes, respectively. After eight cycles of selection and amplification within Pool A, sequences were enriched with a 54\% cleavage efficiency against Hg\(^{2+}\). Similarly, Pool-B was found to catalyze ca. 18\% cleavage efficiency against As\(^{5+}\) after 10 cycles of repeated selection and amplification. The M-fold software analysis resulted in sequences in the two active pools being dominated by “AATTCGGTACGTCCAGTG” and “ATCTCCTCCTGTTC” functional motifs for Hg\(^{2+}\)- and As\(^{5+}\)-based catalysis, respectively. These DNAzymes were found to have higher activity in the presence of transition metal ions compared to alkaline earth metal ions. A maximum cleavage rate of 2.7 min\(^{-1}\) for Hg\(^{2+}\) was found to be highest in our study at a saturating concentration of 500 \(\mu\)M. The results demonstrate that DNAzymes are capable of selectively binding transition metal ions, and catalytic rates are at par with most Mg\(^{2+}\)-dependent nucleic acid enzymes under similar conditions, and indicate their potential as metal-species specific biosensors.

Key words: In vitro selection; DNAzyme; mercury; arsenic; catalytic sequences

INTRODUCTION

DNAzymes or deoxyribozymes have been isolated through in vitro selection (Selective Evolution of Ligands through EXPonential Enrichment-SELEX) protocols (Sen and Geyer, 1998; Joyce, 2004; Fiammengo and Jaschke, 2005). Although so far no deoxyribozymes have been found in nature, a variety of deoxyribozymes have been isolated in vitro that can catalyze different biochemical reactions, including RNA or DNA ligation (Carmi et al., 1998; Cruz et al., 2004), phosphorylation (Li and Breaker, 1999), cleavage of phosphoramidate...
bonds (Burmiester et al., 1997), porphyrin metallation (Li and Sen, 1996), and DNA or RNA cleavage (Carmi et al., 1996; Ting et al., 2004). In addition to their low cost and high stability against chemical and nuclelease degradations, the combination of catalytic activity and substrate recognition ability makes RNA-cleaving DNAzymes attractive reagents for biochemical, environmental, and pharmaceutical applications.

Natural catalysis is often dominated by protein-derived enzymes and, to a lesser extent, by RNA-based enzymes, called ribozymes (Carmi et al., 1996; Lilley, 2005). The relative instability of RNA is attributed to the ribose 2′ hydroxyl functionality, and in the presence of divalent metal cofactors or under alkaline conditions, this hydroxyl forms a 2′ oxyanion that acts as a nucleophile in a transesterification reaction—cleaving the RNA chain (Carmi et al., 1996; Silvermann, 2005). Single-stranded (ss) DNA, although not constrained by the 2′ hydroxyl functionality, was found to catalyze similar reactions to RNA—yet the catalytic capability of ssDNA remains largely unknown. Due to its capability to form structurally and functionally diverse tertiary structures, more than 100 DNAzymes have been isolated from the reactions in vitro. The catalytic efficiency of 10⁹ M⁻¹ min⁻¹ observed for the “10–23” deoxyribozyme isolated by Santoro and Joyce (1997) rivals protein ribonuclease. This deoxyribozyme found to efficiently inhibit the hepatitis B viral RNA (Asahina et al., 1998), abnormal BCR–ABL fusion mRNA (Warashina et al., 1999) and c-myc RNA in vitro (Cairns et al., 1999).

Nucleic acid enzymes, including both ribozymes (made up of RNA that are natural and generated) and deoxyribozymes (made up of DNA that are generated), require a variety of metal ions for catalytic activity, either through facilitation of the folding of RNA and DNA into stable tertiary structures, or through direct participation in the chemical reaction (Pyle, 1993; Liu and Lu, 2003). Compared to protein enzymes, ribozymes utilize a limited number of metal ions. Protein enzymes recruit a variety of metal ions from almost all groups of metals, including the second- and third-period transition metal ions such as molybdenum (Mo) and tungsten (W) (Lippard and Berg, 1994; Holm et al., 1996). The majority of naturally occurring ribozymes is only active in the presence of alkaline earth metal ions and, in some cases, Mn²⁺ (Pyle, 1993; Zivarts et al., 2005). One exception is with the hammerhead ribozyme, which showed a reasonable activity in the presence of Co²⁺, Zn²⁺, and Cd²⁺, although spermine was required for Zn²⁺- and Cd²⁺-catalyzed reactions (Dahm and Uhlenbeck, 1991). Divalent metal ions as cofactors in RNA-cleaving nucleic acid enzymes have been proposed to serve either as Lewis acids (in the form of M²⁺) or as general bases in the form of M(OH)⁺ (Pyle, 1993). Since transition metal ions are generally better Lewis acids, and their hydroxides are usually more basic than alkali and alkaline earth metal hydroxides, transition metal ions might be suitable metal cofactors for DNAzymes. Indeed, many hydrolytic protein enzymes, such as carboxypeptidase, phosphotriesterase, and alkaline phosphatase, use Zn²⁺, Fe²⁺, and Mn²⁺ for catalysis (Coleman, 1992). Transition metal ions are more amenable to spectroscopic studies than alkaline earth metal ions, resulting in better understanding of the structure of metal-binding sites and chemical stability of the interaction.

This work describes the in vitro selection of RNA-cleaving DNAzymes that specifically use transition metal ions (Hg²⁺ and As⁵⁺), which could ultimately lead to development of biorecognition systems (sensors) for a variety of environmentally relevant metal ions. Recruiting transition metal ions may broaden the scope and increase the efficiency of nucleic acid enzyme catalysis.

**EXPERIMENTAL PROTOCOLS**

**Synthesis of oligonucleotides**

Oligonucleotide pools (see Fig. 1) were generated by automated DNA synthesis (Keck Foundation Biotechnology Resource Laboratory, Yale University). Random-sequence DNA libraries were synthesized using an equimolar mixture of four standard phosphoramidites. The prepared DNA oligonucleotides were purified by 10% preparative denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). Fluorescein and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) labels were incorporated into the library during the automated DNA synthesis using Fluorescein-dT amidite and DABCYL-dT amidite (Glen Research, Sterling, VA). Likewise, the adenine ribonucleotide linkage was introduced during solid-state synthesis using A-TOM-CE Phosphoramidite (Glen Research). The TOM protective group on the 2′-hydroxyl group of RNA linkage was removed by incubation with 50 μL of 1 M tetrabutylammonium fluoride in THF at 60°C with shaking for 12 h, followed by addition of 250 μL of 100 mM Tris. Then the mixture was further incubated at 37°C with shaking for 30 min (Mei et al., 2003). The DNA material was recovered using ethanol precipitation and dissolved in water containing 0.01% SDS. *Taq* DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase (PNK) were purchased from Promega (Madison, WI). Cloning was carried out using the TA cloning method (Invitrogen,
Carlsbad, CA). The plasmids having individual catalysts were prepared using a Qiagen MiniPrep Kit. DNA sequencing was performed on a capillary DNA sequencer (Applied Biosystems, Foster City, CA, ABI 3730 DNA Analyzer), following the procedures recommended by the manufacturer.

In vitro selection

In vitro selection scheme of catalytic DNAzymes is depicted in Fig. 2. The 5'-phosphorylated, gel-purified, 90-nt (Pool-A) or 80-nt (Pool-B) random-sequence DNA “X” (100 pmol) was mixed (in an equimolar ratio) with template “T” and acceptor “Y” (Fig. 1). It was further heated to 90°C for 30 s, cooled down to room temperature, and mixed with ligase buffer and T4 DNA ligase (to introduce the modified DNA domain–DNA ligation). Ligation mixture (100 \( \mu \)L) containing 40 mM Tris-HCl, 30 mM NaCl, 10 mM MgCl\(_2\), 1 mg mL\(^{-1}\) bovine serum albumin (BSA), 0.5 mM ATP, and 0.1 Weiss units \( \mu \)L\(^{-1}\) T4 DNA ligase (Mei et al., 2003) was incubated at 25°C for 2 h, and the ligated DNA was purified by 10% denaturing PAGE. The 123-nt (Pool-A) or 113-nt (Pool-B) DNA library synthesized in the above step was used as the initial pool, heated to 90°C for 45 s, cooled down to room temperature, and combined with a 2× selection buffer (100 mM HEPES, pH 6.8 at 23°C, 500 mM NaCl, 300 mM KCl, 10 mM MgCl\(_2\), 10 mM AsHNa\(_2\)O\(_4\), 2.5 mM CdCl\(_2\), 2 mM HgCl\(_2\), 0.5 mM PbCl\(_2\)) to a final DNA concentration of 100 nM. The combined mixture was incubated for self-cleavage reaction at RT for 3 h. We decided to use lower concentrations of As\(^{5+}\), Pb\(^{2+}\), Cd\(^{2+}\), and Hg\(^{2+}\) as the transition metal ions found to be inhibitory on self-phosphorylating DNA. In the case of Mg\(^{2+}\), higher concentration was used since this metal ion did not show any inhibitory effect on activity of the self-phosphorylating DNAzymes (Wang et al., 2002).

As mentioned in Fig. 2 (step 1) a pool of single-stranded 90-nt or 80-nt oligos containing random-sequence nucleotides was first ligated to the acceptor DNA “Y” (23-nt) containing the three key moieties F (fluorophore; Fluorescein-dT), Ar (adenine ribonucleotide), and Q (quencher; DABCYL-dT) as mentioned earlier. The ligated 123-nt or 113-nt DNA was purified by PAGE in step 2. In step 3, the modified DNA molecules were incubated with divalent metal ions for self-cleavage reaction. In the presence of a metal ion, the catalytic DNAzyme carries out the reaction of substrate cleavage at the scissile ribonucleic acid adenosine, resulting in removal of the quencher molecule and thus initiation of fluorescence. This will be measured using fluorescence spectroscopy. The selectivity of DNAzyme was monitored by change in fluorescence (\( \lambda_{ex} = 490 \) nm and \( \lambda_{em} = 520 \) nm). Catalytic DNA molecules cleaving the lone RNA linkage was expected to generate either a 102-nt or 92-nt DNA fragment that could then be isolated by

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**Figure 1.** DNA sequences of Pool-A and Pool-B having library (X), acceptor (Y), and template (T) and the primers used in the in vitro selection protocol.

| Pool-A | Library-(X; 72-nt) | Primer 1: GTGTAACCTGCTGTCG
|GYTCTGTCG
|CGAGCGAAGCT2---AGGCATTTCG
|Template T
|Primer 2: GGTCTGTCGATGTCG--TCCGTAAG
|Primer 3: GGTCTGTCGATGTCGAr

| Pool-B | Library-(X; 62-nt) | Primer 1: GTGTAACCTGCTGTCG
|GYTCTGTCG
|CGAGCGAAGCT2---AGGCATTTCG
|Template T
|Primer 2: GGTCTGTCGATGTCG--TCCGTAAG
|Primer 3: GGTCTGTCGATGTCGAr

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PAGE in step 4. The recovered DNA was amplified by two rounds of polymerase chain reaction (PCR). The first round of PCR was carried out with the use of primers P1 and P2 (step 5). The second round PCR (step 6) used P1 and P3. The double-stranded DNA product produced in the second round of PCR step possessed a single ribonucleotide linkage due to ribo-terminated primer P3. The generated DNA from the second round of PCR was further incubated with NaOH (step 7) to cleave the ribonucleotide linkage (0.3 M NaOH, 90°C, 15 min). The digested DNA mixture was further subjected to purification (by PAGE) and DNA phosphorylation (step 8). DNA phosphorylation was carried by incubation of purified DNA with 10 units of poly nucleotide kinase at 37°C for 1 h in a 100-µL reaction mixture containing 40 mM Tris-HCl (pH 7.8), 30 mM NaCl, 10 mM MgCl2, 1 mg mL⁻¹ BSA, and 0.84 µM ATP. The constructed 5'-phosphorylated DNA was further used to initiate the next round of selection. Likewise, several cycles of selection and amplification were performed to isolate catalytic DNAzymes for specific metal ions by conducting the experiments in presence and absence of metal ions. To facilitate the creation of DNA enzymes, we used Mg²⁺ and several divalent transition metal ions including As⁵⁺, Hg²⁺, and Cd²⁺ in the selection buffer. The total concentration of divalent metal ions was chosen to be 15 mM; individual concentrations as the following: 7.5 mM Mg²⁺, 5 mM As⁵⁺, 1.0 mM Hg²⁺, 1.25 mM Cd²⁺, 0.25 mM Pb²⁺. The different pools were incubated with the same metal ions under similar conditions for all cycles studied. Fluorescence measurements were taken from 100-µL solutions on a HP (1046) fluorescence spectrophotometer. The excitation was set at 490 nm, and emission, at 520 nm. Examination of background fluorescence and control reactions were first measured by adding buffer in to measurement unit.

**Kinetic analysis of catalytic DNAzymes**

The kinetic experiments were carried out by heating DNA in water for 30 s at 90°C, incubating for cleavage in reaction buffer, addition of EDTA to 30 mM to inhibit the reaction, followed by separation of cleavage products by denaturing PAGE. The reaction aliquots were

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**Figure 2.** Selection scheme of catalytic DNAzymes. (1) X DNA was ligated to acceptor DNA Y. (2) The ligated DNA was isolated by polyacrylamide gel electrophoresis (PAGE). (3) Purified DNA was incubated with metal ions to cleave the embedded RNA. (4) The cleavage fragment, Z, was isolated by PAGE. (5) The recovered DNA was amplified by polymerase chain reaction (PCR) using primers P1 and P2. (6) The PCR product from the earlier cycle was reamplified using primers P1 and P3 to introduce a ribonucleotide linkage embedded within DNA. (7) The resulting double-stranded DNAs were treated with NaOH to cleave the RNA linkage. (8) The cleavage fragment is purified by PAGE, phosphorylated, and used in the next cycle.
collected at different time intervals, and the rate constants were measured by plotting the natural logarithm of the fraction of DNA that remains unreacted versus the reaction time (Santoro and Joyce, 1997).

RESULTS AND DISCUSSION

We generated two different starting pools each having $10^{15}$ single-stranded DNA molecules and used the SELEX scheme mentioned in Fig. 2 to select the target specific DNAzymes that can catalyze the RNA cleavage. The protocol developed by Mei et al., (2003) for the in vitro selection of RNA-cleaving DNA catalysts was used in this study. The RNA-cleaving DNAzymes were evolved under different set of conditions. Due to the relative lability of the RNA bond toward hydrolytic cleavage, a ribonucleic adenosine was embedded in the DNA segment. The cleaved DNA was isolated by 10% denaturing PAGE after the cleavage reaction is stopped by the addition of EDTA (pH 8.0) to a final concentration of 30 mM. The isolated cleavage product was amplified by PCR (as mentioned earlier) in a 50-μL reaction volume using primers P1 and P2 (Fig. 1). The amplified DNA product was used as the DNA template for a second PCR reaction using primer P1 and ribo-terminated primer P3 (Fig. 1). The procedures were the same for all selection cycles. The selection stringency was increased during the selection process by decreasing the reaction time and the concentration of available metal ions. A total of 15–25 selection and amplification cycles were carried out with each pool.

**Selection progress and identification of functional sequences**

The selection progress for Pool-A sequences is summarized in Fig. 3. No detectable cleavage activity was observed for Pool-A sequences isolated in generations C0–C3 after the 3-h incubation period. However, significant cleavage was seen in C5. By the C8 cycle, more than 32% of the DNA construct was cleaved after 3-h incubation and ninth cycle yielded drastic increase in catalysis shooting up to ca. 54% of total oligonucleotide pool. At this stage, three more cycles were evaluated to assess further increase in cleavage efficiency, which did not have any effect on ribosomal cleavage (data not shown).

The reaction time and presence/absence of metal ion was then progressively evaluated to isolate very efficient DNAzymes for specific metal ions. The self-cleavage reaction was first allowed only to proceed for 10 min in C10 and 5 min in C11, and the reaction time was further reduced to 1 min in C12 and C13, to 10 s in C14 and C15, and finally to about 1 s in C16–C18. The DNA molecules in C19 were also allowed to react for 1 min. The activity of the selected DNA dramatically decreased from

![Figure 3](image-url)
10 min incubation (C10) to 1-min incubation (C12 and C13) leading to ca. 5 and 7% substrate cleavage, respectively. Further gradual decrease in incubation time to 5 s also produced minimal cleavage averaging ca. 1.5% over three generations (C16–C18). At this stage, the incubation time was increased to 1 min (C19), resulting in a 50% cleavage efficiency. DNA sequences from the 19th cycle (C19) were amplified by PCR and cloned into a vector by the TA cloning method. The plasmids containing the individual catalysts were prepared using a Quiagen MiniPrep kit and sequenced.

The population of molecules obtained after selection and reselection showed surprising similarity in their sequences when they were evolved using in vitro selection. The sequences of 27 individual clones revealed a diverse combination of functional motifs and are classified into three major families based on sequence similarities. Out of 27 mercury-active sequences, 16 fell in to one family (Family-Im), exhibiting “AATTCCGTAGGTCGACGT” as a conserved region. The remaining 11 sequences could be classified in two different families (Family - Im and IIIm) of seven and four sequences, respectively (Fig. 4).

A secondary structure for the above-mentioned sequence (most common structural motif) was predicted by the M-fold program (Zuker, 2003) and on the basis of this structure, we designed a trans-cleaving enzyme (called Mer27) and examined further for its metal ion specificity and kinetics as mentioned below. Most of these sequences were found to be highly active in the presence of Hg$^{2+}$ than in the presence of Mg$^{2+}$, As$^{5+}$, Cd$^{2+}$, or Pb$^{2+}$ when tested individually. The confirmation of its catalytic activity and the specificity was established in the following experiments.

The results obtained from Pool-B sequences (Fig. 5) were found to be different from what was noticed with Pool-A. No cleavage was observed until the eighth cycle (C8) and progressively increased to 18% at C11 without further improvement even after three more cycles (C12–C14). When the incubation time was reduced to 10 min (C15), 5 min (C16), 1 min (C17–C18), 10 s (C19–C20), and finally 1 s (C21–C22) at 22nd cycle—over eight cycles—the decrease in ribosomal cleavage to 2% was noticed at the lowest incubation time (1 s). When increased to 1 min in the 23rd cycle, an increase in ri-

![Figure 4](Image)

Figure 4. Mercury active sequences that were isolated after the 19th cycle of in vitro selection and few representative secondary structures from each family of sequences predicted by M-fold software.
bosomal cleavage to 14% was observed similar to Pool-A. DNA sequences from the 23rd generation were amplified, cloned into a vector by the TA cloning method, and sequenced as mentioned earlier.

The functional molecules for Pool-B obtained from M-fold program (after selection and reselection) showed minimal dissimilarity leading to two families based on sequence similarities (Fig. 6). The sequences of 17 individual clones revealed a major conserved motive “ATCTCCTCCTGTTC” found in 12 clones (family-Ia). The remaining five clones fell in to one family (Family-IIa). Also, the most common structural motif (called Ars-17) was engineered in to a trans-cleaving enzyme as mentioned above, and examined further for its metal ion specificity and kinetics. The activities found in both pools were lost once the respective metal ions were omitted from the reaction mixture.

In vitro selection techniques rely on the probability that some DNA molecules in a random-sequence library fold into an appropriate tertiary structure with lowest possible energy and catalyze a desired reaction (in this case, RNA cleaving). Catalytic activity is derived from the, formation of 2’,3’-cyclic phosphate and 5’-hydroxyl RNA termini from the RNA 2’ hydroxyl group on the adjacent phosphodiester linkage. Considering the probability that one or more DNA molecules in a vast random-sequence pool will possess some catalytic propensity for a given chemical transformation, the extent of sequence diversity in the DNA pool may have a profound effect on the final outcome of the experiment. Without sufficient sequence diversity, the probability of finding a proficient DNA enzyme can be unfavorably low. Thus, sufficient sequence diversity in the DNA pool (and, hence, more catalytic DNA sequences) is a prerequisite for the successful isolation of efficient deoxyribozymes. The catalytic sequence diversity established by in vitro selection is governed largely by the choice of selection pressures, one of which is the length of the reaction time. Schlosser and Li (2004) examined the effect of reaction stringency (in terms of shortening of the reaction time) during in vitro selection of RNA-cleaving DNAzymes and found a logarithmic decrease in sequence diversity with decrease in reaction time. In vitro selection of RNA-cleaving DNAzymes in the above-mentioned study resulting in 43 sequences in a 5-h reaction period, compared to eight sequences when the reaction period decreased to 5 s. In contrast, we found 27 and 17 different sequences for mercury (II) and arsenic (V), respectively, when we incubated for 1-s reaction time, indicating efficient selection and higher sequence diversity.

Catalytic activity and metal ion specificities

In vitro selected nucleic acid enzymes were previously found to be active in the presence of metal ions other
than the metal ions used during selection (Faulhammer and Famulok 1997; Li et al., 2000). It was demonstrated here that divalent metal ions were necessary for the activity of RNA cleavage as no more than 0.8% of the substrate was cleaved after 50 h in the presence of 100 μM EDTA. To further investigate this catalytic activity and the cleavage specificity, two selected DNAzymes, Mer-27 and Ars-17, were assayed in the presence of different metal ions. These cloned mercury-active and arsenic-active sequences were randomly chosen and sampled for self-cleavage activity. Under the in vitro selection conditions mentioned above, highly abundant Mer-27 and Ars-17 were individually examined, and they showed high cleavage activity against the target metal species (0.09 and 0.05 min⁻¹, respectively). These two cloned sequences were found at highest frequency of occurrence in their respective pools. Further, these DNAzymes were characterized in terms of their catalytic rate by conducting the experiments with individual metal ions. The catalytic rates of Mer-27 and Ars-17 (in the presence of their target metal Hg²⁺ and As⁵⁺) is presented in Fig. 7 in terms of fluorescence relative to background values (F/F₀). Similar plots were obtained for all metal ions to derive the first-order reaction rate (min⁻¹) and the catalytic rates range from 0.006 to 0.01 min⁻¹. The following activity trends were observed based on the data presented in Table 1.

**Mer-27**: Hg²⁺ > > Cu²⁺ ~ Zn²⁺ ~ Pb²⁺ ~ Cd²⁺

> Ni²⁺ > Mg²⁺ ~ Ca²⁺ > Pd²⁺ ~ As⁵⁺

**Ars-17**: As⁵⁺ > Cd²⁺ > Hg²⁺ ~ Pb²⁺ > Cu²⁺

> Zn²⁺ > Ni²⁺ > Mg²⁺ ~ Ca²⁺ > Pd²⁺

The degree of dissimilarity observed for the two different pools examined under similar conditions was surprising considering that selection was carried out under the same combinations of metal ions. At 100 μM concentration, Mer-27 showed highest activity in presence of Hg²⁺ followed by Cu²⁺ and Ars-17 was found to be highly active in presence of As⁵⁺ followed by Cd²⁺. The high selectivity of Mer-27 toward Hg²⁺ followed by Cu²⁺ has been described elsewhere. Nolan and Lippard (2003) reported an in vitro selection protocol for the development of fluorescent sensors for mercuric ion in the presence of other ions with the exception of Cu²⁺, which effectively competed with Hg²⁺ binding. Thomas et al. (2004) studied the inhibition of mercuric ion on the cleavage rate of an imidazole modified, M²⁺-dependent self-cleaving 9₂₅-11 DNA system. They found that inhibition
of cleavage is highly selective in the presence of Hg\textsuperscript{2+} with the exception of Cu\textsuperscript{2+}.

From Table 1 it can be noted that arsenic-active DNAzymes were also found to exhibit reasonably high activity in presence of Cd\textsuperscript{2+}. This type of multiple metal ion activity has been reported earlier. For example, the 8–17 DNAzyme selected from solutions containing Mg\textsuperscript{2+} (Santoro and Joyce, 1997), also exhibited activity against Mg\textsuperscript{2+}/histidine (Faulhammer and Famulok, 1997) and As\textsuperscript{5+} (for Ars-17) in 100 mM HEPES buffer at pH 7.0. However, it was shown to be the most active in the presence of Pb\textsuperscript{2+}, with activity decreasing in the following order: Pb\textsuperscript{2+} > Zn\textsuperscript{2+} > Ca\textsuperscript{2+} > Mg\textsuperscript{2+}. Similarly, Liu et al. (2003) studied the activity of a DNAzyme (DEC22-18) selected using Co\textsuperscript{2+} against other metals, and noticed that it is highly active in the presence of Mn\textsuperscript{2+}, Cd\textsuperscript{2+}, and Ni\textsuperscript{2+} as well. This versatility of metallodeoxyribozymes may be attributable to the fact that metal solutions are contaminated with trace quantities of nontargeted metals with similar ionic radii and pKa values in water for their respective hydrated forms. Yet, this correlation is not noticed with all metal ions studied. Similar results have been reported for hammerhead ribozymes (Dahm et al., 1993), 10–23 deoxyribozymes (Santoro and Joyce, 1998), and 17E deoxyribozyme (Li et al., 2000).

Table 1. Catalytic activities of Mer-27 and Ars-17 in the presence of metal ions at 100 μM concentration.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>K\textsubscript{obs} (min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mer-27</td>
</tr>
<tr>
<td>Hg\textsuperscript{2+}</td>
<td>1.90</td>
</tr>
<tr>
<td>Cu\textsuperscript{2+}</td>
<td>1.22</td>
</tr>
<tr>
<td>Zn\textsuperscript{2+}</td>
<td>1.15</td>
</tr>
<tr>
<td>Pb\textsuperscript{2+}</td>
<td>0.55</td>
</tr>
<tr>
<td>Cd\textsuperscript{2+}</td>
<td>0.50</td>
</tr>
<tr>
<td>Ni\textsuperscript{2+}</td>
<td>0.21</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>0.020</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>0.022</td>
</tr>
<tr>
<td>As\textsuperscript{5+}</td>
<td>0.007</td>
</tr>
<tr>
<td>Pd\textsuperscript{2+}</td>
<td>0.01</td>
</tr>
</tbody>
</table>

In addition to playing structural roles in the DNAzyme, the DNAzyme activity may also be influenced by the direct involvement of the divalent metal ions in chemical reactions. For example, the metal hydride can act as a general base and deprotonate the 2′-hydroxyl at the cleavage site. Alternatively, the metal ion can serve as a Lewis acid by direct coordination to the oxygen of 2′-hydroxyl, thereby weakening the 2′-O—H bond (Silverman, 2004). Metal ions may further coordinate to the nonbridging
phosphodiester oxygen to either make the phosphorus center more susceptible to nucleophilic attack, or help stabilize the developing negative charge of the oxy-anion in the trigonal-bipyrimal transition state. In addition, direct coordination of divalent metal ions to the 5'-oxygen leaving group will stabilize the developing negative charge and accelerate the cleavage of 5'-O—P bond. The inverse correlation between the pKₐ value of the metal ligands and the ribosomal cleavage efficiency of Mer-27 and Ars-17 provides evidence for metal ions playing some of these catalytic roles as indicated by Kawakami et al. (2000).

In summary, Pool-A resulted in the selection of highly mercury-active sequences, while Pool-B was found to produce arsenic-active sequences. It is interesting to note that transition metals are favored over alkaline earth metals. This increased preference of transition metal ions over alkaline earth metals may indicate differences in the metal binding sites on the DNAzymes, including the binding affinity, ligand-set, ionic-radii, and geometry as reported by Bruesehoff et al. (2002). Alkaline earth metal ions tend to maintain their hydration state and bind nucleic acids through outer-sphere coordination with low to moderate binding affinity (Holbrook et al., 1977). However, transition metal ions like Zn²⁺, Co³⁺, Mn²⁺, and Cd²⁺ can bind to both the nonbridging phosphate oxygen and the O or N groups on the nucleic acid bases using either inner-sphere or outer-sphere coordination.

Concentration dependency

The effect of metal ion concentration on the catalytic cleavage by Mer-27 and Ars-17 was examined by conducting the experiments with increasing metal ion concentrations (10–500 μM). Figure 8 indicates that the Mer-27 cleavage rate increases 10-fold, from 0.3 to 2.7 min⁻¹, while the Ars-17 rate increased from 0.19 to 1.7 min⁻¹. The rates observed at the highest concentration evaluated (500 μM) are in agreement with earlier reported literature values (Liu et al., 2003; Ting et al., 2004). Liu et al. (2003) studied a variety of RNA-cleaving DNAzymes at different pH and found that the most of them showed fairly large rate constants ranging from 0.2 to 1.3 min⁻¹. Particularly, the Mer-27 is one of the fastest DNAzymes reported to date. The significantly high cleavage activity is probably due to the balanced and appropriately positioned metal binding sites, or the preference of metal ions with a favorable pKa of metal bound water or a better Lewis acidity or all together can play in random or organized fashion. The formation of an RNA 2',3'-cyclic phosphate via intramolecular cleavage is facile even without catalysis, as incubations of an RNA strand under basic conditions lead to random scission along the entire length of the strand. The major difference between earlier studies and this study is the use of unmodified nucleotides for selection of DNAzymes. Nolan and Lippard (2003) developed a water-soluble, turn-on sensor using sulfur incorporated (3,9-dithia-6-azaundacane moiety) DNA pool that exhibits high selectivity and sensitivity for Hg (II).

Similarly, Thomas et al. (2004) selected high-affinity DNAzyme-based ligand for sensing Hg²⁺ using imidazole-modified DNA. On the contrary, the present approach, herein, makes use of routine DNA material (having no such modifications) for the development of highly selective and specific DNAzymes for mercury (II) and arsenic (V).

**SUMMARY**

The isolation of two distinct families of DNAzymes that recognize Hg²⁺ and As⁵⁺ resulted in the emergence of new functional motifs with high catalytic rates (2.6 min⁻¹ for Mer-27 and 1.7 min⁻¹ for Ars-17). Independent of whether DNAzymes exist in nature, it may now be possible to create a wide range of engineered DNA enzymes for application as highly stable biocatalysts. The information obtained in this study for metal-specific DNAzymes can be used more efficiently to understand sequence, structural, and functional configurations of DNA folding, and hence design of effective DNAzyme-based biosensors for a variety of environmentally relevant metal ligands. Diffusion of these systems into the practical realm will require immobilization of DNAzymes onto solid supports as biorecognition elements while maintaining specificity and sensitivity in environmentally relevant matrices.
RNA-CLEAVING DNAZYMES

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