DOI: 10.1002/cbic.200800632

Biochemical Characterization of a Uranyl Ion-Specific DNAzyme

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The biochemical characterization of a DNAzyme that is highly specific for uranyl (UO_2^{2+}) ions is described. Sequence alignment, enzyme truncation, and mutation studies have resulted in a conserved sequence that folds into a bulged stem–loop structure. Interestingly, a G·A pair next to the scissile site is important for the uranyl ion-specific DNAzyme; this is reminiscent of the G·T wobble base pair adjacent to the cleavage site that is crucial for the Pb^{II}-specific 8–17 DNAzyme activity. Therefore wobble pairs might be important for formation of metal-specific metal-binding sites in DNAzymes. The DNAzyme binds the uranyl ion with a dissociation constant of 469 nM, which is among the strongest metal-binding affinities in nucleic acid enzymes reported to date. This explains why a catalytic beacon fluorescent sensor based on this enzyme has a detection limit (45 pM) that rivals the most-sensitive analytical instrument. It also has over 1 000 000-fold specificity for the uranyl ion over other metal ions. The DNAzyme can carry out multiple turnover reactions that follow the Michaelis–Menten equation, with a k_{cat} of 1.46 min^{-1} and a K_M of 463 nM, similar to that of the 8–17 DNAzyme. The pH profile shows a bell-shaped curve that reaches a maximum at pH 5.5, at which the in vitro selection was carried out; this suggests that in vitro selection can be performed to obtain DNAzymes with optimal performance under specific conditions under which practical applications are required. These findings enrich our fundamental understanding of metal-binding sites in nucleic acids and allow the design of sensors with better performance.

Introduction

In the early 1980s, RNAs were discovered to perform enzymatic reactions and thus called ribozymes.[1,2] This discovery was followed by the finding in 1994 that DNAs can also function as enzymes, thus named deoxyribozymes or DNAzymes.[3] Biochemical studies of ribozymes and DNAzymes indicate that they require divalent metal ions for efficient catalysis; this suggests that nucleic acid enzymes can form specific metal-binding sites.[4–7] Although most early studies on these enzymes employed Mg^{II} as the metal cofactor, many research groups, including ours, also pursued the possibility of using transition-metal ions, and many transition-metal-specific nucleic acid enzymes have been obtained, thanks mainly to the development of a combinatorial method called in vitro selection.[4,8–12] For example, in vitro selection has resulted in DNAzymes that are specific for Pb^{II},[3,13,14] Co^{II},[15] Mn^{II},[16] Zn^{II},[17] Hg^{II},[18] and Cu^{II}.[19–21] These experiments firmly established that nucleic acid enzymes can bind metal ions specifically, just like metalloprotein enzymes.[4] Compared to Mg^{II}-dependent enzymes, enzymes employing transition-metal cofactors usually have much higher metal-binding affinities. For several enzymes, the metal-binding affinity and specificity are so high that they were used as components for metal-ion biosensors, such as Pb^{II}, Cu^{II}, Hg^{II}, and Mn^{II} sensors.[22–27] These successes led us to purposefully employ in vitro selection to obtain metal-specific DNAzymes for metal-sensing applications. For example, we have recently selected an RNA-cleaving DNAzyme that is active in the presence of UO_2^{2+}.[28]

Uranium is a naturally occurring radionuclide.[29] Uranium has recently been significantly enriched to generate energy and make nuclear weapons, and its use is likely to grow as an important energy source. Because enriched uranium has been, or has the potential to be released into the environment, a method for simple, fast, on-site, and real-time detection and quantification of uranium might be helpful in environmental remediation and in minimizing human exposure to uranium and its related adverse health effects, such as radiation and kidney damage. The uranyl ion (UO_2^{2+}) is the most stable chemical form of uranium in water and is therefore highly bioavailable and poses the greatest risk to human health.[30,31] To search for DNAzymes that can recognize uranium, an in vitro selection experiment was carried out by using UO_2^{2+} as the metal cofactor,[28] and one of the selected DNAzymes was engineered into a fluorescent biosensor for uranium detection with a detection limit of 45 pM and a selectivity of more than 1 000 000-fold higher for uranyl over any other tested metal ion.[28]

Even though the above-described DNAzyme has been shown to bind the uranyl ion with high specificity, we know very little about the conserved sequence and biochemical features of the enzyme. To gain deeper insights on the basic properties of the uranyl-specific DNAzyme, and to help design

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better sensors, we herein report characterization of the DNAzyme in terms of sequence alignment, enzyme mutation, metal-binding affinity, specificity, multiple turnover properties, and pH profile. A comparison with the Pb^{II}-specific 8–17 DNAzyme is also presented.

Results and Discussion

Sequence alignment and secondary structure analysis

To isolate uranyl-specific RNA-cleaving DNAzymes, eleven rounds of in vitro selection were carried out, and the UO_2^{2+} concentration was gradually decreased from 5 mM to 100 μM UO_2^{2+}, as described previously.[28] Table 1 shows the metal concentration and incubation time for each round. The selections were performed in pH 5.5, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer with 250 mM $NaNO_3$. The uranyl acetate stock solution was made in a 2× sodium citrate solution, and subsequent dilutions were made with water. As can be observed from Table 1, the performance of the pool dropped at round 11 compared to that of round 10. Therefore, the round 10 DNA was cloned and sequenced, and 86 sequences were obtained. Clone no. 39 was chosen for UO_2^{2+}-sensing applications.[28] The trans-cleaving 39E DNAzyme system contains a substrate strand (39S, Figure 1 A) and an enzyme strand (39E). Substrate strand 39S is a RNA/DNA chimera with a single RNA linkage (rA) that serves as the cleavage site, and 39E recognizes 39S through two Watson–Crick base-pairing regions. The DNAzyme can be folded into a bulged three-way-junction structure (Figure 1 A).

To analyze the sequence requirement for catalysis, the enzyme strand in the catalytic core region was divided into three parts as outlined in Figure 1 A: the three-nucleotide noncanonical base-pairing region 1, the stem loop region 2, and the bulged region 3. Among the 86 sequences that were obtained, 68 of them were aligned and the rest were abandoned due to the presence of large fractions of truncation or stretches of unidentifiable nucleotides. To relate the sequence information with function, alignment was performed based on the secondary structure of 39E and only the sequences close to the catalytic core were presented (Figure 1 D). It needs to be pointed out here that no reselection was performed and the alignment was based on the initial selection. Among the 68 sequences, seven have activity lower than 0.01 min^{-1} and were not analyzed further. For the remaining 61 sequences, interestingly, 49 of them can be aligned against the secondary structure of 39E; this suggests that the uranyl-specific DNAzyme has a highly conserved metal-binding site that can recruit the uranyl ion for catalysis. The cleavage

Table 1. Reaction conditions for selection of uranium specific DNAzymes.

Round(s)	Selection conditions [UO_2^{2+}]	Selection conditions Incubation time	Assay [% cleavage in 2 h] 1 mM UO_2^{2+}	Assay [% cleavage in 2 h] 0.1 mM UO_2^{2+}
1–5	5 mM	5 h	–	–
6[a]	5 mM	5 h	65	–
7	5 mM	1 h	70	70
8	1 mM	1 h	78	83
9	1 mM	30 min.	75	90
10	0.1 mM	30 min.	78	90
11	0.1 mM	15 min.	65	72

[a] One round of negative selection with 500 μM Pb^{II} (1 h) was incorporated after round 6.

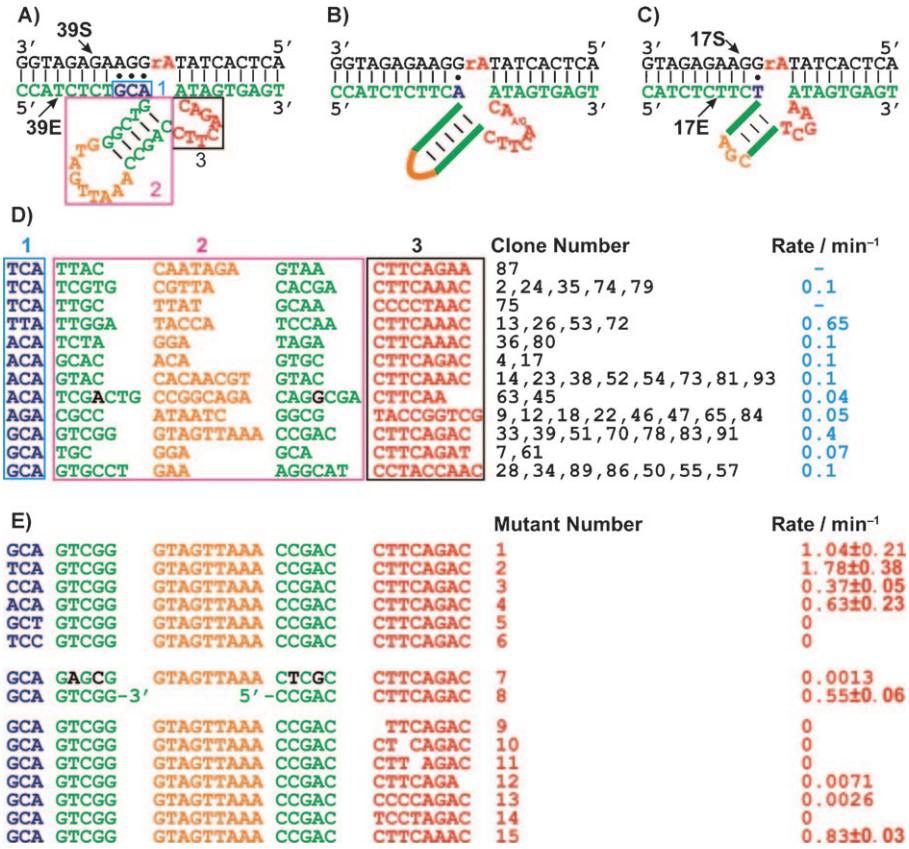

Figure 1. Secondary structure and sequence alignment of the in-vitro-selected uranyl ion-dependent DNAzymes. A) Secondary structure of the 39E DNAzyme and conserved sequences of B) the uranium DNAzymes and C) lead specific 17E DNAzyme are shown. Sequence alignment and activity of the uranium DNAzymes in D) the cis-cleaving form right after selection and in E) the trans-cleaving form after sequence truncation. The nucleotides that are marked in black in region 2 of D) and E) indicate mismatched base pairs in the stem. The activities of clone 87 and 75 were not measured.

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rate constants of each clone right after selection without any modification are shown in Figure 1 D.

We further investigated the sequence requirements for regions 1 to 3 separately by constructing mutated trans-cleaving enzymes based on 39E (Figure 1 E). 39E has a sequence of GCA in region 1 (Figure 1 A) that does not form perfect base-pairing interactions with the corresponding substrate part. From the sequence alignment (Figure 1 D), it can be observed that in addition to G, T and A were also present at the first position. Because this position is highly tolerable to mutations, it should not be crucial for catalysis. To identify the optimal DNAzyme, the first nucleotide in the region was systematically mutated to C, A, and T (mutant 2–4, Figure 1 E), while keeping all the remaining nucleotides unchanged. The cleavage rates for the mutated enzymes were compared, and a result of T > (G, A, C) was obtained. From this result, it can be concluded that substrate recognition is the role of this position. The highest rate constant was obtained when the T·A base pair was formed. For the second position, C is the most frequently encountered base in the aligned sequence, although T can also give very high activity. Therefore, this position is also for substrate binding. The third position is a highly conserved adenine. Mutation of this adenine to a thymine or cytosine (Figure 1 E, mutants 5, 6), for example, completely abolished the activity of the DNAzyme.

It is interesting to compare this result with that for the Pb^{II}-specific 8–17 DNAzyme (Figure 1 C); while a G·T wobble base pair adjacent to the cleavage site is crucial for the 8–17 DNAzyme,[13] the G·A pair is important for the 39E DNAzyme. It is known that noncanonical base pairs are crucial for the structure and function of RNA, such as those in tRNA and ribozymes.[32–34] While wobble pairs do not appear to be present for several other important DNAzymes such as the 10–23 DNAzyme and mutated 8–17 DNAzymes that were recently reported by Li et al.,[35], our results suggest that noncanonical pairs are useful for tuning the DNAzyme activity and might be important for formation of specific metal-binding sites in DNA.[36] Further biophysical experiments will be performed to understand metal binding, including X-ray crystallography, NMR spectroscopy, and fluorescence resonance energy transfer (FRET).[37–40]

It can be observed from the sequence alignment in Figure 1 D that the stem–loop structure (region 2 in Figure 1 A) is highly conserved, although its size can vary significantly. The stem contains three to six base pairs in the cloned sequences, and the loop size varies from three to nine nucleotides. This suggests that the stem loop might not be specific for catalysis and might play only a structural role. To confirm this hypothesis, several mutated enzymes were further tested. In one experiment, the loop was deleted and the enzyme was split into two halves. This split DNAzyme (mutant 8, Figure 1 E) can still cleave the substrate efficiently with a rate constant of 0.55 min^{-1}, which is comparable to the original 39E. Introducing mismatches into the stem region, however, significantly decreased the activity of the DNAzyme. For example, in mutant 7, two mismatches were introduced to the stem, and the activity was only 0.0013 min^{-1}. Such a replaceable stem–loop structure has proven to be a useful scaffold for engineering allosteric DNAzymes or aptazymes.[25]

As can be observed from Figure 1 D, the nucleotides in region 3 of the enzyme strand are also highly conserved; most are octamer loops. The first four nucleotides are rich in pyrimidines with a sequence of CTTC for most enzymes. Although CCCC is also active with a significantly decreased cleavage rate (mutant 13), TCCT is totally inactive (mutant **14**). The next three nucleotides are rich in purines, and the last one is a cytidine for most sequences, although a thymine is also active. Insertions and deletions in this region are also observed from the sequence alignment, and generally significant drops in activity were seen with such modifications. Based on the sequence alignment and mutation studies, the essential sequence in the DNAzyme is shown in Figure 1 B. The bars that are connected by short dashes indicate Watson–Crick base-pairing regions, and as long as the base pairs are maintained, the nucleotide content can be changed without significantly affecting activity. One of the most optimal DNAzyme secondary structures is presented in the Supporting Information.

Uranyl ion-dependent activities

Because the 39E DNAzyme has been shown to be highly effective in detecting uranium with a dynamic range from 45 pM to 400 nM,[28] it is important to study the enzyme activity as a function of UO_2^{2+} concentration. The study was performed under single-turnover conditions with approximately 1 nM ^{32}P-labeled 39S and 2 μM 39E. As can be observed from Figure 2 A, the enzyme showed a bell-shaped response with increasing UO_2^{2+} concentration, and the highest activity was observed with approximately 5 μM UO_2^{2+}. To determine the apparent dissociation constant (K_d) for uranium binding, three independ-

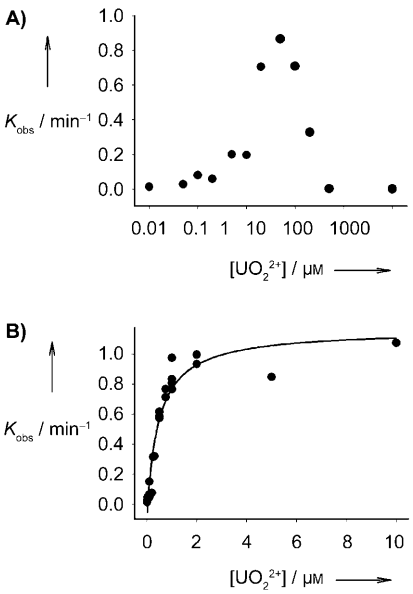

Figure 2. UO_2^{2+}-dependent activity of the 39E DNAzyme. A) Assays were performed with UO_2^{2+} concentrations from 10 nM to 1 mM. B) Assays were performed in a metal concentration range in which activity increased with increasing UO_2^{2+}.

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ent experiments were performed at the low-metal-concentration region (Figure 2B). The K_d for UO_2^{2+} was calculated to be 469 ± 96 nM, and k_{max} was determined to be 1.23 ± 0.07 min^{-1}. This K_d value was approximately fourfold more than that obtained from fluorescence assays,[28] thus suggesting weaker affinity when measured using gel-based assay. This discrepancy could be explained by the fact that in the fluorescent assay the enzyme concentration was only 50 nM, while in the gel-based assay the enzyme concentration was 2 μM, and the excess amount of enzyme might bind the uranyl ion and thus decrease the effective concentration of the free uranyl ion. To confirm this, the activity assay was performed with varying concentrations of the enzyme while fixing the substrate and uranyl ion concentrations. An approximately four-fold decrease in the rate constant was observed when the enzyme concentration was increased from 1 to 10 μM.

The apparent binding affinity of 39E for UO_2^{2+} is relatively strong compared to the affinity of other DNAzymes for divalent metal ions. For example, the K_d values of 8–17 DNAzyme for Pb^{II} and Zn^{II} are 15.4 μM and 0.997 mM, respectively,[13,14] the K_d of the leadzyme for Pb^{II} is approximately 5 μM,[41] and the K_d values of the 10–23 DNAzyme for Mg^{II}, Ca^{II}, and Mn^{II} are 180 mM, 60 mM, and 2.6 mM, respectively.[42,43] This strong affinity explains why the uranyl ion sensor has the highest sensitivity among DNAzyme-based metal ion sensors. As it is a hard Lewis acid, the uranyl ion has a high affinity towards the phosphate oxygen, and its binding constant with DNA is on the order of 10^{10} M^{-1} at pH 4.[44] Therefore, the very low K_d of the DNAzyme is not unexpected.

Such bell-shaped responses of enzymatic activity with increasing metal concentration were also reported for several other DNAzymes.[18,19,45] In one case, the inhibitory effect at high metal concentrations was attributed to the disruption of DNA base-pairing interactions by nonspecific metal/DNA interactions.[45] To understand the mechanism of inhibition in the current uranium DNAzyme, we tested the activity of the Pb^{II}-specific DNAzyme (Figure 1C) in the presence of 100 μM uranium, and similar inhibition effects were also observed (data not shown). Therefore, the inhibition was attributed to the nonspecific interaction between high concentrations of the uranyl ion and DNA.

pH profile

A pH-dependent study of 39E in the presence of UO_2^{2+} was performed to provide mechanistic insights and to determine the optimal condition for UO_2^{2+} detection. The activity of 39E was first examined over a broad pH range from 3.5 to 7.0 under single-turnover assay conditions. The DNAzyme was completely inactive at pH 3.5. As shown in Figure 3A, the pH curve for 39E is biphasic, increasing approximately linearly from pH 4.0 to 5.5 and then decreasing almost linearly to pH 7.0. Interestingly, the optimal pH of 5.5 is exactly the same as the pH at which the in vitro selection was carried out. These results further confirmed that in vitro selection can be tailored to obtain optimal DNAzymes under real-world conditions.

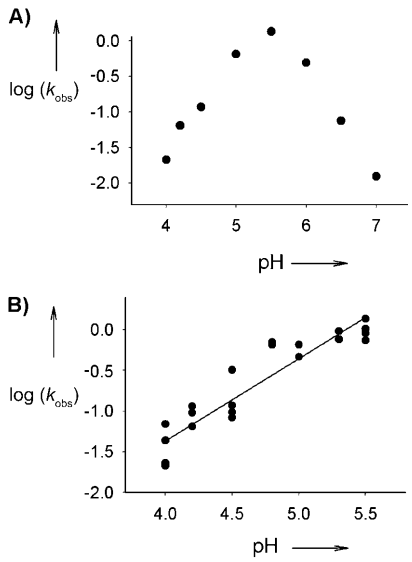

Figure 3. pH dependence of 39E under single-turnover conditions. Assays were performed in 50 mM NaOAc (pH 4.0–5.0), MES (pH 5.5–6.5), or HEPES (pH 7.0). Assays were performed in A) a wide pH range or B) in the low pH region.

This biphasic pH-dependent behavior has been observed in other nucleic acid enzymes. Li and co-workers also observed a similar trend for several DNAzymes selected at different pH values.[16] Most of the DNAzymes were dependent on divalent metal ions and had the highest activity at or near the pH of the buffer used during the selection. pH-dependent activities were also reported for a Cu^{II}-dependent DNA-cleaving DNAzyme.[45] A similar behavior in the leadzyme was attributed to the formation of insoluble Pb^{II} hydroxide species at higher pH.[41] The same could be true of the uranyl DNAzyme; however, the poor solubility of UO_2^{2+} cannot account for all of the observed trends because precipitation should not result in a linear decrease of the rate constant with pH with a slope of −1. The most likely explanation of the result is that pH influences the uranyl ion speciation in solution and pH 5.5 produced a uranyl species that is the most effective cofactor for carrying out the reaction. At pH values higher than 5, hydrolyzed species such as $UO_2(OH)^+$ start to dominate,[46] which might not be the optimal cofactor for catalysis. Indeed, one of the advantages to in vitro selection is that the selected DNAzyme might be able to distinguish between very similar chemical species. Future work will be performed to test whether the DNAzyme reacts with other uranium species such as UO_2^+. More careful studies were performed at a pH below 5.5, and a linear relationship between pH and $\log k_{obs}$ was obtained with a slope of approximately 1 (Figure 3B); this is suggestive of the presence of a single deprotonation step in the rate-limiting step of the catalytic process.

Multiple-turnover reactions

To test whether the 39E DNAzyme can perform multiple-turnover reactions, assays were carried out by using a fixed 4.3 nM

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39E enzyme and varying the FAM-labeled substrate concentration from 250 nM to 4 μM. Three independent measurements were carried out, and the results are shown in Figure 4. The initial rate of cleavage (v_0) was plotted, and the data were fit to the Michaelis–Menten equation. A K_M of 463 nM and a k_{cat} of 1.46 min^{-1} were obtained. This result indicates that the enzyme is a real catalyst and can perform multiple-turnover reactions. The K_M value is comparable to that obtained for the 8–17 DNAzyme (K_M = 650 nM in 50 mM MES, pH 6.0 with 100 μM Zn^{II}). As shown in Figure 1, these two DNAzymes have very similar substrate-binding arms and therefore similar K_M values are expected. In this experiment, saturated UO_2^{2+} concentration (4 μM) was used.

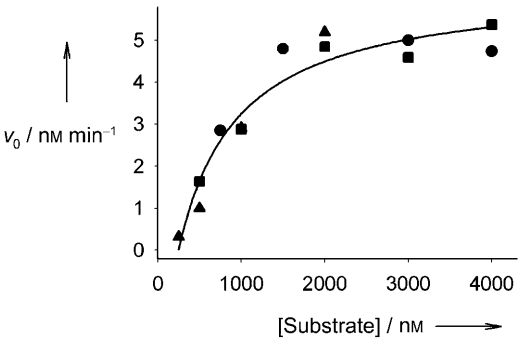

Figure 4. Multiple-turnover property of the 39E DNAzyme. Three independent assays were performed as indicated by different shapes in the data points.

Metal specificity studies

We previously showed that the sensor had over 1 000 000-fold selectivity for uranium over any other metal ions in the fluorescence-based assay, and only uranium induced a fast fluorescence increase. Here we investigate whether such high selectivity is due to the intrinsic preference for using UO_2^{2+} as a cofactor for cleavage or due to artifacts of metal–fluorophore interactions such as quenching. A 5′-fluorophore-labeled 39S was used as the substrate and the 39E enzyme was unmodified. Nineteen different metal salts ($AgNO_3$, $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, $VOSO_4$, $Mn(OAc)_2$, $Fe(NH_4)_2(SO_4)_2$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, $Cd(ClO_4)_2$, $Hg(ClO_4)_2$, $Pb(NO_3)_2$, $TbCl_3$, $EuCl_3$, and $Th(NO_3)_4$) were added at three different concentrations (1 μM, 20 μM, and 1 mM), and the reactions were allowed to run for 15 min at room temperature. Cleavage products were detected by denaturing gel electrophoresis. Among all the conditions tested, only 1 mM Pb^{II} showed approximately 0.7% cleavage after 15 min, which is approximately 1 000 000 times slower than cleavage in the presence of uranium. Other metal ions were even slower compared to Pb^{II}. These data confirmed that the lack of fluorescence increase in the sensor was mainly due to the lack of cleavage in the presence of other metal ions. The above-described metal specificity assays indicate that the enzyme is not active in the presence of Mg^{II} even at a millimolar concentration level. This lack of Mg^{II}-dependent activity is different from that of the 8–17 DNAzyme; even though the 8–17 DNAzyme is the most active in the presence of Pb^{II}, it is still active in the presence of millimolar concentration of Mg^{II}. A comparison of the secondary structures of 39E (Figure 1 B) and 8–17 DNAzymes (Figure 1 C) suggests that while the stem loop, particularly the "AGC" sequence in the stem loop, is required for the 8–17 DNAzyme activity,[13] such a stem loop is not needed for 39E DNAzyme activity.

Effect of citrate

It is interesting to note that while the 39E DNAzyme activity was completely inhibited when the UO_2^{2+} concentration was higher than 100 μM, the lowest UO_2^{2+} concentration used in the selection process was 100 μM. The reason that highly sensitive UO_2^{2+} DNAzymes can still be selected under such inhibiting conditions was attributed to the presence of 2× citrate in the selection buffer. Citrate is known to form complexes with the uranyl ion and the free UO_2^{2+} concentration in the selection buffer would be much lower.[47,48] Indeed, when citrate was added to the fluorescent uranium sensor, the sensor response went down dramatically as shown in Figure 5.

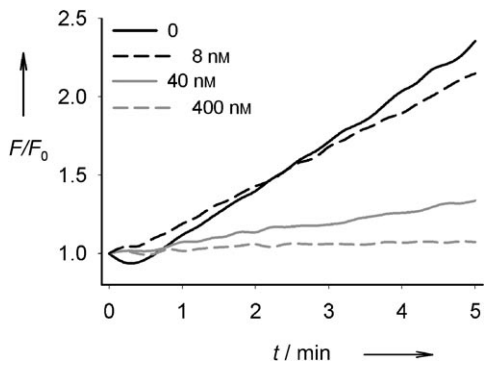

Figure 5. The response of the 39E-based fluorescent uranium sensor in the presence of 4 nM UO_2^{2+} with varying concentrations of citrate (see legend for citrate concentration). The y axis is the ratio of fluorescence enhancement. The protocol for the experiment is described in a previous publication.[28]

Conclusions

In conclusion, we have characterized a uranyl ion-specific DNAzyme in terms of sequence alignment, enzyme mutation, metal-binding affinity, specificity, multiple turnover properties and pH-rate profile. Because the G·A pair next to the scissile site is found to be important for the uranyl ion-specific DNAzyme, similar to the G·T wobble base pair in the Pb^{II}-specific 8–17 DNAzyme, wobble pairs might be important for formation of metal-specific metal-binding sites. While many DNAzyme selections have been performed with alkaline earth metals such as Mg^{II} and Ca^{II} and transition metals such as Zn^{II}, Co^{II}, Mn^{II}, Cu^{II}, and Pb^{II} as cofactors, this is the first example of using an actinide as a metal cofactor. It further demonstrates that the selection method can be broadly applied to many metal ions of choice. The extremely high sensitivity and selectivity of the

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fluorescent uranyl ion sensor can be traced to the strong uranyl ion binding affinity and specificity. The optimal pH for the DNAzyme activity matches the pH at which in vitro selection was carried out, further demonstrating that in vitro selections can be tailored to obtain DNAzymes with optimal performance at specific conditions where practical applications are required.

Experimental Section

Materials: All DNA samples were purchased from Integrated DNA Technologies (Coralville, IA, USA) and were purified by polyacrylamide gel electrophoresis (PAGE) or HPLC. Uranium acetate dihydrate was purchased from Fisher Scientific and was dissolved in a 50 mM stock solution in water or in sodium citrate (100 mM). Dilutions were made in water. Procedures for selection were described in a previous publication and are not repeated here.[28] Except for the in vitro selection experiment, most biochemical assays were performed in the absence of citrate. To make the MES buffer (2-[*N*-morpholino]ethane sulfonic acid), solutions of ultrapure MES monohydrate and MES sodium salt (from Sigma) were made with Millipore water (500 mM). The solutions were treated overnight with Chelex beads (iminodiacetic acid, from Sigma) to remove any divalent metal ions present. The solutions were then mixed to obtain the proper pH, and the resulting buffer was then filtered with a syringe filter (0.2 μm).

Biochemical assays: The 39S substrate was 5′-labeled with $\gamma AT^{32}P$ (GE Healthcare) by using T4 kinase from Invitrogen. The enzyme and substrate were annealed in a water bath (65 °C) for 2 min and were then cooled to room temperature in 20 min to equilibrate to that temperature. All assays were performed at room temperature. Single-turnover assays were conducted with ^{32}P-labeled substrate (final concentration approximately 1 nM). The final concentration of enzyme was 2 μM for all assays, unless indicated otherwise. MES (50 mM, pH 5.5) with $NaNO_3$ (300 mM). was used as a buffer. After annealing the solution of enzyme and substrate and allowing the solutions to equilibrate to room temperature, an aliquot (2 μL) of the solution was taken out and added to stop solution (10 μL, 50 mM EDTA, 8 M urea, 90 mM Tris, 90 mM boric acid, 0.05% xylene cyanol, and 0.05% bromophenol blue) as a control to indicate the extent of cleavage before the reaction was initiated. The reaction was initiated by addition of an equal volume of a solution of UO_2^{2+} to the enzyme–substrate solution. At various predetermined time-points, an aliquot (2 μL) of the reaction mixture was added to stop solution (10 μL) in a 96-well plate. The uncleaved substrate and cleaved products were separated by denaturing polyacrylamide gel electrophoresis (20%) and quantified by a Molecular Dynamics Storm 430 phosphorimager (from Amersham Biosciences). The percent product at time t, $\%P_t$, was calculated by dividing the amount of cleavage product by the total amount of substrate plus product, after subtraction of background radioactivity in the gel. Kinetic curves were plotted by using SigmaPlot and fit to Equation (1):

$$\%P_t = \%P_0 + \%P_\infty(1-e^{-kt}) \quad (1)$$

in which $\%P_0$ is the initial percent product (at $t=0$), $\%P_t$ is the percent product at the endpoint of the reaction (t) , and k is the rate constant of cleavage. The enzyme-dependent activities were analyzed by plotting k_{obs} (in min^{-1}) versus enzyme concentration and fitting the resulting curve to Equation (2)

$$k_{obs} = k_{cat}[E]/(K_M' + [E]) \quad (2)$$

in which k_{cat} is the second-order rate constant, and k_{obs} is the pseudo-first-order rate constant under steady-state conditions. K_M' is the Michaelis–Menten constant that was determined under single-turnover conditions. For the pH-dependent enzyme activity study, $\log k_{obs}$ was plotted as a function of pH. The data points were fit to a line ($y=ax+b$) where a is the slope of the line.

Acknowledgements

This work has been supported by the Office of Science (BER), U. S. Department of Energy, Grant No. DE-FG02-08ER64568, and the U. S. National Science Foundation (CTS-0120978, DMR-0117792, and DMI-0328162).

Keywords: bioinorganic chemistry · biotechnology · DNA cleavage · DNAzymes · selection · uranyl ion

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Received: September 22, 2008
Published online on January 13, 2009

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