Reselection Yielding a Smaller and More Active Silver-Specific DNAzyme

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Supporting Information

ABSTRACT: Ag10c is a recently reported RNA-cleaving DNAzyme obtained from in vitro selection. Its cleavage activity selectively requires Ag⁺ ions, and thus it has been used as a sensor for Ag⁺ detection. However, the previous selection yielded very limited information regarding its sequence requirement, since only ~0.1% of the population in the final library were related to Ag10c and most other sequences were inactive. In this work, we performed a reselection by randomizing the 19 important nucleotides in Ag10c in such a way that a purine has an equal chance of being A or G, whereas a pyrimidine has an equal chance of being T or C. The round 3 library of the reselection was carefully analyzed and a statistic understanding of the relative importance of each nucleotide was obtained. At the same time, a more active mutant was identified, containing two mutated nucleotides. Further analysis indicated new base pairs leading to an enzyme with smaller catalytic loops but with ~200% activity of the original Ag10c, and also excellent selectivity for Ag⁺. Therefore, a more active mutant of Ag10c was obtained and further truncations were successfully performed, which might be better candidates for developing new biosensors for silver. A deeper biochemical understanding was also obtained using this reselection method.

INTRODUCTION

Silver is an important metal for technology, but it is also a heavy metal contaminant in the environment.⁶ Ag⁺ has very interesting roles in interacting with nucleic acids. The most well-known example is the specific binding of Ag⁺ by cytosine forming a C–Ag⁺–C base pair.³ In fact, Ag⁺ can bind to all of the DNA bases, and DNA is a popular template for preparing fluorescent silver nanoclusters.⁴,⁵

Although this simple C–Ag⁺–C interaction allowed rational design of silver-binding DNAzymes, we are interested in exploring other binding mechanisms, which may have even better specificity. In general, aptamer selections have been performed for various metal ions.⁶–⁹ However, few high-quality aptamers are available for metal binding. Another method is to select DNA based on catalytic activity. Catalytically active DNA sequences are called DNAzymes,¹⁰–¹⁴ and the most extensively studied DNAzymes perform the RNA cleavage reaction.¹⁰,¹¹,¹³–¹⁵,¹⁷ Interestingly, most DNAzymes require metal ions for activity.¹⁸,¹⁹ Using DNAzymes, many metal ions can be detected with excellent sensitivity and specificity,²⁰–²² such as Na⁺,²³–²⁶ Ca²⁺,²⁷,²⁸ Pb²⁺,²⁹ Zn²⁺,³⁰–³² UO₂²⁻,³³ Hg²⁺,³⁴ Cd²⁺,³⁵ Cu²⁺,³⁶ and trivalent lanthanides.³⁷,³⁸ We recently used Ag⁺ for selection and obtained a highly Ag⁺-specific DNAzyme named Ag10c.³⁹

Selection of unmodified DNAzymes for strongly thiophilic metals has been quite challenging. Normally modified bases,³⁰,³⁴ or modified phosphate backbones, were needed.³⁵,³⁶ This can be attributed to the poor interactions between the DNA phosphate and thiophilic metals,¹⁸,⁴⁰ yet such interactions are critical for the RNA cleavage reaction. At the same time, strong interactions between DNA bases and thiophilic metals can lead to DNA misfolding,⁴¹ which also hinders DNA selection. Interestingly, the Ag10c DNAzyme did not contain any modification, but our final selection library had very poor activity.³⁹ The success of our previous selection was largely due to the deep sequencing technology, allowing us to identify a very small fraction of active sequences (~0.1%).

With this single active sequence, the information on its conserved nucleotides is limited.⁴² We have made some rational single-nucleotide mutations, but none of the mutants worked better than the original Ag10c.⁴³ We suspect that there might be more active mutants that require mutation of multiple nucleotides at the same time, but this can hardly be achieved by rational mutations. Herein, we performed a
separation of the active strand. A polymer spacer (the black diamond) stops the polymerase, producing two strands of DNA of unequal lengths for subsequent reselection experiment by partially randomizing the Ag10c sequence. Indeed, we found a more active mutant containing multiple mutations, and this has led to a smaller catalytic core and deeper insights into this DNAzyme.

## MATERIALS AND METHODS

### Chemicals.

The DNA samples were purchased from Integrated DNA Technologies (Coralville, IA) and Eurofins (Huntsville, AL). AgNO₃ and other metal salts were from Sigma-Aldrich at the highest available purity. Sodium acetate, sodium chloride, ammonium acetate, tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)-ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid, and ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate were from Bio Basic (Markham, Ontario, Canada). Sso Fast EvaGreen supermix was from Bio-Rad. T4 DNA ligase, deoxynucleotide (dNTP) mix, gel loading dye (6×), Taq DNA polymerase, and low-molecular-weight DNA ligase were from New England Biolabs.

### In Vitro Selection.

The selection was carried out according to our previous report with some minor modifications. Brieﬂy, the initial library was synthesized by ligating Lib-FAM with Lib-rA in the presence of the splint DNA (see Table 1 for DNA sequences). For the cleavage reaction, a ﬁnal Ag⁺ concentration of 10 μM was added to the DNA library in buffer A (50 mM MES, pH 6.0, 50 mM NaNO₃) with 60 min of incubation at room temperature. Then, the reaction was terminated by 8 M urea and puriﬁed with 10% denaturing polyacrylamide gel electrophoresis (dPAGE). The position corresponding to the cleavage product was excised from the gel. The DNA was extracted with buffer B (1 mM EDTA, 10 mM Tris—HCl, pH 7.0), and further puriﬁed with a Sep-Pak C18 column (Waters). After drying in an Eppendorf vacufuge at 30 °C overnight, the dried DNA was re-suspended in 50 μL of Milli-Q water. A small fraction of this DNA was ampliﬁed by two polymerase chain reaction (PCR) steps using previously described thermocycling protocols.

### Sequencing.

Prior to deep sequencing, the round 3 and round 4 libraries and the naive library before selection were subjected to PCR1, as described above. The library from this step was subjected to another PCR using the forward primer (P701) and the reverse primer (P501), each containing a unique index sequence (see Table 1). The PCR products were puriﬁed with 2% agarose gel and extracted using a gel extraction kit (IBI Scientiﬁc). The extracted DNAs were eluted in 30 μL of Milli-Q water, and their concentrations were quantiﬁed using a NanoDrop spectrophotometer to be between 10 and 20 ng/μL. The sequencing was performed at the McMaster Genomics Facility (Hamilton, ON).
Activity Assay. For cleavage activity assays, a final of 10 μM metal ions were incubated with 0.5 μM DNAzyme complex in buffer A for 60 min (unless noted otherwise) at room temperature. The complex was formed by annealing the FAM-substrate (see Table 1) and the enzyme in buffer A. The reaction was quenched with 8 M urea and separated on a 15% dPAGE at 120 V for 80 min. The gel bands were quantified with a ChemiDoc MP imaging system (Bio-Rad).

Results and Discussion

Reselection. The structure of the Ag10c DNAzyme is shown in Figure 1A.39,43 Its substrate strand has a single RNA linkage (rA for ribo-adenine) serving as the cleavage site, whereas its enzyme strand has a hairpin with two single-stranded bulges. The important nucleotides for Ag+ recognition are in these bulges,43 and these nucleotides were numbered. For our original selection, we obtained only around 50 copies of Ag10c out of the 50 000 total sequences.39 Most of the other tested sequences were inactive. Therefore, the information from single mutations was still limited. If more active double or triple mutations exist, the individual point mutation studies would miss them.

To address this problem, we decided to partially randomize the library to perform a reselection. We kept the overall secondary structure of Ag10c and made changes only to the 19 important nucleotides (numbered in Figure 1A). In addition to the 17 in the loop, two more were also included (18 and 19) to increase flexibility near the cleavage site. If an original base is a G or A, in the reselection library, it had 50% chance of being G and 50% being A. The same was applied to the pyrimidines, where C and T were randomized to C/T with an equal possibility. We kept the hairpin to force the library to fold into such a secondary structure. As base pairs are formed between purines and pyrimidines, such a library design may offer insights into some base pairing interactions that were previously overlooked.

The reselection was performed as schematically drawn in Figure 1B. After incubating with Ag+ for the cleavage reaction, the library was loaded into a gel, and the cleaved sequences (74-nucleotide) were harvested and amplified by two steps of PCR to go back to the original library for the next round of selection.39 We observed a steady growth of the cleavage yield in the first three rounds (Figure 2A), whereas little further increase was observed in round 4. Since a goal of the reselection was to study the sequence requirement for activity,
we hoped to retain a high diversity of the library. Therefore, we stopped at round 4 and sequenced the naive library as well as the round 3 and 4 libraries.

**Sequence Analysis.** The sequences were aligned using the Geneious software, and the most populated 100 families were examined in detail. For the round 4 library, about 85% of the sequences were a single motif, and we named it AgB0 (Figure 2B, red bars). A careful analysis of AgB0 indicated that it is similar to the Na⁺-specific NaA43 DNAzyme (Figure 2C, the first two rows),\(^{23}\) and also to a DNAzyme reported by us in another work.\(^{26}\) NaA43 is active in the presence of Na⁺ alone, and it is not surprising that we also obtained similar sequences, since we also had Na⁺ in our buffer. Selection of recurring sequences is quite common for in vitro selection,\(^{44}\) and one of the well-known examples is the 8−17 motif that has been commonly seen in divalent-metal-dependent selections.\(^{31,45−48}\) Here, we used monovalent metals to avoid the 8−17,\(^{49}\) but the NaA43 appeared to be another such sequence. As the round 4 library was dominated by AgB0, we did not study it further.

We then turned our attention to the round 3 library, which contained a total of 10⁵ sequences. Indeed, both the expected Ag10c-like sequences (AgB1 to AgB10 in Figure 2C), and also AgB0, were found. We plotted the distribution of these sequences in Figure 2B (black bars). The disappearance of Ag10c and growing of AgB0 were observed from round 3 to 4. We also analyzed the naive library before selection, and the sequences were random (data not shown).

As the round 3 library contained a large fraction of Ag10c-like sequences, we focused on it. In the round 3 library, we obtained 1341 of the original Ag10c sequence, which was about 1% of the total library. In this work, our reselection library was specially designed. Normally, reselection is performed by retaining 70% of the original base at each position, while it has 10% possibility of being one of the other three nucleotides. This way, it is expected that the majority of the sequence is the original one. For 19 randomized bases, the original sequence should be \((0.7)^{19} = 0.11\)% of the original library. In our case, we only made purine-to-purine and pyrimidine-to-pyrimidine randomization, and the original Ag10c was only \((0.5)^{19} = 0.00019\)% or 1.9 ppm of the original sequence. If the library was not enriched, the Ag10c should only have 0.19 copy from the 100 000 total DNA sequences. The fact that we had over 1000 copies of Ag10c indicated its selective enrichment, and it appeared that the Ag10c is already an optimized DNAzyme.

**Important Nucleotides.** Aside from Ag10c, the next two families (AgB1 and AgB2) each had over 1000 sequences, and they contained two and three mutations, respectively. From AgB3 to AgB8, these sequences had only single mutations. To have a full statistic understanding, the distribution of each nucleotide in the final library was calculated, which can give insights into the tolerance of each nucleotide to mutation (Table 2). Some interesting observations were made. First, most positions were dominated by one base (e.g., >95% possibility), suggesting those positions do not tolerate purine−purine or pyrimidine−pyrimidine mutations. A few positions, such as 5, 9, and 19, had ~30% of chance of such mutation. One position even had a few percent of pyrimidine-to-purine mutation (e.g., position 5). Therefore, these positions might not be important for catalysis.

Positions 5 and 9 are close to the hairpin, and it is easy to understand that they might be unimportant. Position 19 is within the substrate-binding arm. It has 71.3% chance of being
an adenine, which can form a normal base pair with the substrate region (Figure 1A). When it is a guanine (the rest 28.7%), it can still form a wobble with the substrate. Interestingly, the position 18 thymine was fully retained and no cytosine was detected at this position. Therefore, its base pairing with the cleavage site adenine might be critical for activity. Overall, this is a highly conserved DNAzyme. The loop sequences from positions 1–4 and 10–18 are around 95% or higher conserved.

Figure 3. Gel-based activity assay of cleavage of the FAM-labeled substrate hybridized with (A) AgB1 or AgB2; (B) AgB9 or AgB10; and (C) Ag10c DNAzymes. The reaction was performed with various concentrations of Ag⁺ in buffer A (50 mM MES, pH 6.0 with 50 mM NaNO₃) for 60 min. (D) Metal selectivity assays using the AgB1 DNAzyme with 10 and 100 μM of various metal ions. Inset: A gel micrograph showing the cleavage with 10 μM metals.

Figure 4. Catalytic core sequences of (A) Ag10c, (B) AgB1, (C) AgB1-del-A7, (E) AgB1b, (F) AgB1c, and (G) AgB1d DNAzymes. (D) Kinetics of the cleavage of AgB1-del-A7 and Ag10c. Inset: A gel of AgB1 cleavage. (H) Cleavage rates of a few DNAzymes with a further shortened hairpin. The reaction was performed in pH 7.5 buffer with 10 μM Ag⁺ and 200 mM NaNO₃.
Activity Assays. The above sequence analysis was based on statistics. To confirm the analysis, we then tested individual DNAzyme sequences. Most of the sequences contained only single mutations, and their activity has already been previously studied. Therefore, this work focused on sequences with more than one mutation. Out of these aligned sequences, the first two most abundant families are quite similar. AgB1 has two mutations compared to Ag10c (namely TSC and A9G mutations). AgB2 has these same two mutations and an additional one. This extra mutation did not seem to be important, since it is in the substrate-binding arm. We measured their activities using a FAM-labeled substrate (Figure 3A). Ag+-dependent cleavage was observed from 5 to 20 μM of Ag+, and the yield appeared to be even higher than that of the wild-type Ag10c (Figure 3C). The other sequences with extensive mutations are AgB9 and AgB10. However, these two sequences were inactive in the presence of Ag+ (Figure 3B). A key feature of the Ag10c DNAzyme is its high selectivity for Ag+. Therefore, the AgB1 mutant was also assayed with 27 monovalent, divalent, and trivalent metal ions (Figure 3D), and only Ag+ produced cleavage. This selected DNAzyme also showed higher activity with a higher Na+ concentration, and this property is unique to the Ag10c DNAzyme. Therefore, the selected DNAzyme is likely to have the same cleavage mechanism.

Smaller but Faster DNAzyme. Because AgB1 and AgB2 appeared to be more active than Ag10c and they also retained excellent selectivity for Ag+, we wanted to rationalize the improved activity. These two sequences both contained TSC and A9G mutations. A careful examination of the sequences revealed that very few sequences had just one of these mutations, and this suggests that these two nucleotides need to covariate. Therefore, we suspected that these two positions might form a base pair (e.g., before mutation it forms an A–T base pair, and after mutation it becomes a more stable G–C pair, Figure 4A,B). Considering its neighboring positions may also form a base pair (G6 and C8), we suspect these hypothetic base pairs could be part of the hairpin with just an extra adenine (A7). To test this hypothesis, we designed a sequence with the A7 deleted (Figure 4C).

We first compared the rate of DNAzyme cleavage. The AgB1-del-A7 was highly active (Figure 4D), and the cleavage appeared to reach saturation in 5 min. Therefore, A7 can indeed be deleted. We then quantitatively fitted the cleavage kinetics and a first-order rate of 0.80 min⁻¹ was obtained for the AgB1-del-A7 (Figure 4E). For comparison, the original Ag10c was only 0.38 min⁻¹ under the same condition. Therefore, we obtained a more active mutant through this reselection experiment. A rate of 0.80 min⁻¹ is the highest among RNA-cleaving DNAzymes without a polyvalent metal ion. For comparison, the i-histidine-dependent DNAzyme (0.2 min⁻¹ with 100 mM histidine), the NaA43 DNAzyme (0.1 min⁻¹ with 400 mM Na⁺), and the EtNa DNAzyme (0.1 min⁻¹ with 2 M Na⁺) were all much slower.

After deleting A7, two extra base pairs can nicely form, and the true catalytic core region (e.g., the two loops) becomes smaller. The right side of the loop has only T1 to G4, and the left side has only G10 to G17. Therefore, we have reduced the core region by five nucleotides. This will make it easier for subsequent biochemical and biophysical studies. At the same time, the faster mutant will also be analytically useful.

To further test our hypothesis, we deleted C5, G6, C8, and G9 based on AgB1-del-A7 and obtained a truncated DNAzyme named AgB1b (Figure 4E), which essentially shortened the stem region by two base pairs. It is still quite active with a rate of around 0.4 min⁻¹ (Figure 4H, the red bar). This experiment also confirmed that these four deleted bases did not participate in the catalytic reaction, and they mainly function by base pairing. We then further truncated the stem to leave only four base pairs (Figure 4F), and were finally left with only eight bases for the whole hairpin (Figure 4G). In both cases, the activity remained, although slightly lower than that of the original AgB1-del-A7 (Figure 4H). With these truncations, we have significantly decreased the size of the DNAzyme, and it will make further mechanistic and structural biology studies easier.

CONCLUSIONS

In summary, on the basis of the previously reported Ag10c DNAzyme, we performed a reselection experiment with the intention to understand its sequence requirement and to search for more active mutants. On the basis of the reselection, we obtained a statistic understanding of the importance of each nucleotide in the catalytic loop of Ag10c. In particular, a covariating double mutant was identified allowing us to discover a new base paired region. On the basis of this understanding, the enzyme activity improved by 1-fold and the sequence can also be shortened significantly. This mutant could be a more active candidate for further development of biosensors for Ag+.

Our reselection library contained only purine–purine and pyrimidine–pyrimidine randomization, and this type of simple reselection library could be a way to obtain interesting information. Such a reselection library is cost-effective to synthesize, and it has provided interesting insights about this Ag10c example to optimize its secondary structure. A future step is to design a fully randomized library with each position mutated to all four nucleotides based on this shortened DNAzyme. This two-step reselection method may also be applicable to studies of other DNAzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02039.

- Salt-concentration-dependent cleavage activity (PDF)

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Notes

The authors declare no competing financial interest.

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