

DNA-Catalyzed Reductive Amination**

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Deoxyribozymes are particular DNA sequences that have catalytic ability, analogous to protein enzymes as functional amino acid sequences.^[1,2] The discovery of natural RNA enzymes (ribozymes) spurred the search for artificial deoxyribozymes,^[3] which are identified by *in vitro* selection.^[4] Most deoxyribozyme-catalyzed reactions involve phosphodiester bond cleavage or ligation,^[5–7] although other reactions such as Diels–Alder reaction^[8] and thymine dimer photoreversion^[9] have also been reported.

We have initiated a comprehensive effort to identify deoxyribozymes that catalyze reactions of amino acid side chains, initially focusing on small peptide substrates and with the longer-term goal of covalently modifying large proteins.^[10] Towards this goal, as part of a recent study we examined the ability of several new deoxyribozymes to modify tyrosine (or serine) side chains of tripeptide substrates that are not covalently tethered to the deoxyribozyme, although the tether was required to enable the selection process.^[11] Deoxyribozymes identified using tethered peptide substrates were also active with untethered peptide substrates, but with substantially lower rate and yield. Therefore, in new experiments, here we performed *in vitro* selection directly using untethered peptide substrates. This effort required a modified selection procedure that was intended to involve reductive amination as a step merely to “capture” catalytically active DNA sequences. Surprisingly, this selection process provided deoxyribozymes that operate entirely independently of the tripeptide substrate and instead catalyze a Ni²⁺-dependent reductive amination involving the N²-amine of a guanosine nucleobase on an RNA substrate, which reacts with an oligonucleotide-dialdehyde substrate. Reductive amination is a key biochemical process, for example, for amino acid biosynthesis and catabolism using amino acid dehydrogenases (oxidases)^[12] and transaminases (aminotransferases).^[13] *In vitro*, enzymatic reductive amination is important in both laboratory-scale^[14] and industrial-scale organic synthesis.^[15] Reductive amination may also have been important in RNA World scenarios.^[16,17] Our unexpected discovery of DNA-

catalyzed reductive amination suggests further exploration of the abilities of nucleic acid enzymes to catalyze this interesting and potentially useful class of reaction.

For any *in vitro* selection strategy that does not involve physical compartmentalization or segregation of individual candidate sequences,^[18] the selection design must include a mechanism by which individual, functional sequences become separable from the vast excess of nonfunctional sequences. Importantly, this mechanism must operate in parallel fashion without requiring independent interrogation (i.e., screening) of each candidate sequence, which is impractical for the typical populations of >10¹⁴ sequences. Although many *in vitro* selections have relied upon binding of functional sequences to a solid support (bead), typically through biotin–streptavidin interactions, our efforts have generally relied upon polyacrylamide gel electrophoresis (PAGE) shift.^[5] A key advantage of the gel-shift approach is that functional sequences are separated largely on the basis of oligonucleotide length (size), which has increased or decreased substantially upon successful reaction. In contrast, bead-based methods are blind to the length of binding sequences and are therefore subject to artifacts that are avoided in the gel-shift approach.

Shown in Figure 1a is the particular selection strategy used in our recent report for identifying deoxyribozymes that covalently modify the tyrosine (or serine) side chain of an oligonucleotide-anchored, tethered tripeptide substrate.^[11] The resulting deoxyribozymes function by catalyzing Tyr (or Ser) side chain reaction with a 5'-triphosphorylated RNA substrate, leading to a Tyr-RNA (or Ser-RNA) nucleopeptide linkage. The tether is mandatory to enable the selection process; functional DNA sequences append the oligonucleotide anchor to themselves upon covalently modifying the anchored tripeptide, thereby leading to a gel shift that enables their separation. The new deoxyribozymes were subsequently evaluated with untethered substrates lacking the oligonucleotide anchor. In all cases greatly reduced rate and yield were observed, indicating that these deoxyribozymes depend strongly on the oligonucleotide anchor of the tripeptide substrate for optimal function.^[11]

Omitting the oligonucleotide anchor and directly using an untethered peptide during the selection process would not induce a substantial gel shift for catalytically active DNA sequences, because the small peptide mass is insufficient in this regard. Therefore, seeking to retain gel shift as the physical basis of selection, here we designed the two-stage strategy shown in Figure 1b. First, an untethered peptide was used during the selection step, which—in accord with our recent report^[11]—included both Mg²⁺ and Mn²⁺ cations as available catalytic cofactors (50 mM HEPES, pH 7.5, 40 mM MgCl₂, 20 mM MnCl₂, 150 mM NaCl, 2 mM KCl, 37°C, 14 h). Second, in the “capture” step, any deoxyribozymes that

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[**] This research was supported by grants to S.K.S. by the National Institutes of Health (grant number GM065966), the Defense Threat Reduction Agency (grant number BRBA08-L-2-0001), and the National Science Foundation (grant number 0842534). We are grateful to the anonymous reviewers and Amit Sachdeva for their insightful comments and experimental suggestions.



Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201104976>.

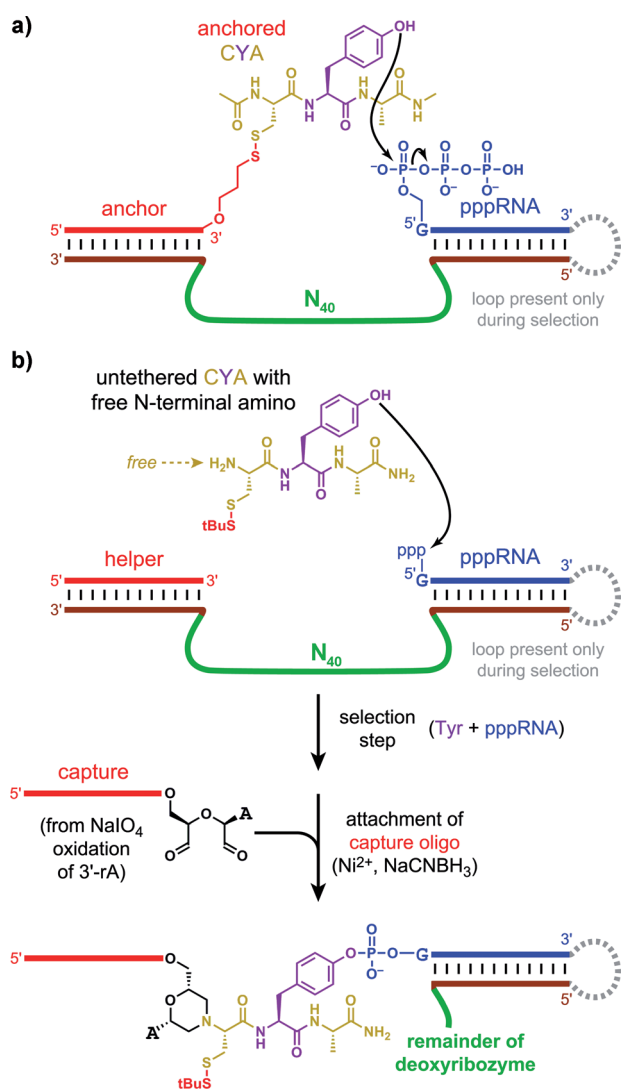


Figure 1. In vitro selection strategies. a) Previous strategy for identifying DNA-catalyzed modification of a Tyr side chain of an oligonucleotide-anchored peptide substrate.^[11] b) Strategy employed here, intending to identify DNA-catalyzed modification of a free peptide substrate. The free peptide is first allowed to react with the 5'-triphosphorylated RNA. Active deoxyribozymes are then captured by reaction of the peptide N-terminal α -amino group with the 3'-terminus of a NaIO_4 -oxidized capture oligonucleotide.

successfully appended the tripeptide to themselves through reaction of the tripeptide with the RNA substrate 5'-terminus were separated by gel shift through attachment of a capture oligonucleotide. This attachment used reductive amination to join the tripeptide N-terminal α -amino group with the NaIO_4 -oxidized dialdehyde terminus of the capture oligonucleotide (which is entirely DNA, except for a 3'-terminal ribonucleotide). This capture step was performed in 100 mM NaOAc, pH 5.2, 50 mM NiCl_2 , and 10 mM NaCNBH_3 at 37 °C for 14 h, as optimized to reproducible $\approx 50\%$ yield using independently prepared substrate standards (data not shown).

The new selection strategy shown in Figure 1b was implemented using our well-developed general selection

methodology,^[5,19] with the addition of the capture step involving the NaIO_4 -oxidized capture oligonucleotide, Ni^{2+} , and NaCNBH_3 . After 8 rounds using the untethered CYA substrate, the yield provided by the uncloned selection pool was 36%. However, despite the selection design, assay of the round 8 uncloned pool revealed that the observed reactivity was tripeptide-independent, indicating that any DNA-catalyzed reaction did not involve the tripeptide. In contrast, reactivity was entirely dependent upon both Ni^{2+} and NaCNBH_3 as well as the presence of the NaIO_4 -oxidized capture oligonucleotide, unexpectedly yet strongly suggesting that a DNA-catalyzed reductive amination process was occurring.

The round 8 selection pool was cloned, and individual deoxyribozymes were analyzed. Seven distinct DNA enzyme sequences were studied in more detail (see Figure S1 in the Supporting Information for sequences). One of the new DNA enzymes, named 8QA124, formed its product by catalyzing reaction of the NaIO_4 -oxidized capture oligonucleotide with the RNA substrate in 60–90% yield with k_{obs} of 0.37 h^{-1} ($t_{1/2} = 1.7 \text{ h}$; Figure 2a). The other deoxyribozymes had yields ranging from 18–55% after 22 h (see Figure S2 in the Supporting Information). In control experiments, no product was observed ($< 0.2\%$ yield in 22 h) when the deoxyribozyme was replaced with a noncatalytic template strand that has 40 random nucleotides (N_{40}) or when the deoxyribozyme strand was omitted altogether (untemplated). When a template strand was included that replaced N_{40} with zero, one, or two unpaired T nucleotides (T_0 , T_1 , T_2), the yield in 22 h was only 0.6, 0.4, or 0.2%, respectively. From the T_0 result (0.6% in 22 h = $2.7 \times 10^{-4} \text{ h}^{-1}$), a conservative lower limit for the 8QA124 rate enhancement was calculated as 1400.

All seven of the new deoxyribozymes functioned well when the RNA substrate was 5'-monophosphate rather than 5'-triphosphate, indicating that the triphosphate was not the site of electrophilic reactivity as originally intended (see Figure S3 in the Supporting Information; six of the seven deoxyribozymes including 8QA124 catalyzed $< 1\%$ reaction involving the 5'-hydroxyl RNA substrate). Moreover, in all cases the reaction strictly required each of NaIO_4 (for the capture oligonucleotide), Ni^{2+} , and NaCNBH_3 (see Figure S4 in the Supporting Information). At this point, we tentatively concluded that the DNA-catalyzed reactions each involved attack of an amine nucleophile from the RNA substrate into the NaIO_4 -oxidized dialdehyde terminus of the capture oligonucleotide (Figure 2b). This is the opposite “polarity” of reaction as originally intended for the capture step; in the observed reaction an amino group of the RNA serves as the nucleophile, rather than the 5'-triphosphate of the RNA acting as the electrophile as originally intended (Figure 1b).

In support of this conclusion, MALDI-TOF mass spectrometry of each deoxyribozyme's reaction product was consistent with a single reductive amination reaction between an RNA nucleobase exocyclic amine and the dialdehyde moiety of the capture oligonucleotide (Figure 3 and Table S1 in the Supporting Information). The MS data were inconsistent with a second reductive amination by the same nitrogen atom to form a morpholine ring. Treatment of each reaction product with RNase T1 (which cleaves specifically to

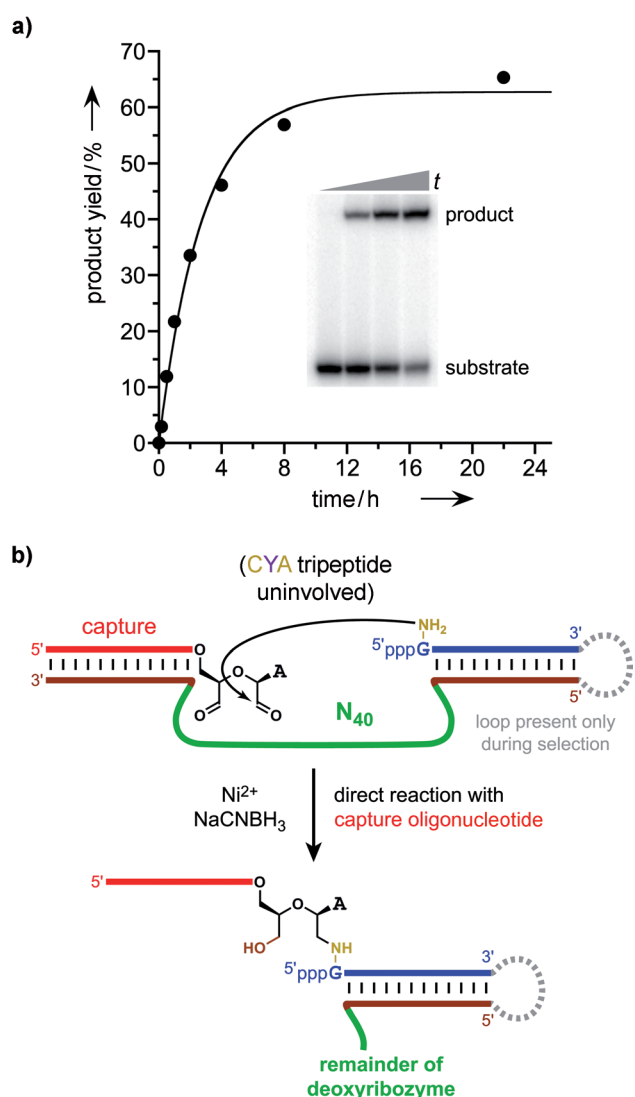


Figure 2. Catalysis by the 8QA124 deoxyribozyme, and proposed DNA-catalyzed reductive amination reaction involving an amine nucleophile on the RNA substrate reacting with an aldehyde on the capture oligonucleotide. a) Catalysis by 8QA124. The RNA substrate was 3',32P-radiolabeled. Incubation conditions: 100 mM NaOAc, pH 5.2, 50 mM NiCl₂, and 10 mM NaCNBH₃ at 37 °C. b) Proposed reductive amination reaction catalyzed by 8QA124 and the other newly identified deoxyribozymes. The second aldehyde group of the product is shown as reduced to an alcohol on the basis of additional experimental data (see text).

the 3'-side of guanosine residues) led to a truncated product that corresponds to cleavage after G2 of the RNA sequence. Together, these data compel the assignment of each deoxyribozyme's reaction product as involving reductive amination between the N²-amine of G1 of the RNA substrate and the dialdehyde terminus of the capture oligonucleotide, as depicted in Figure 3a. Consistent with this conclusion, replacement of G1 of the RNA substrate with inosine, whose hypoxanthine nucleobase lacks the N²-amine of guanine, completely suppressed the catalytic activity of all seven deoxyribozymes (see Figure S5 in the Supporting Information). In further support of this conclusion, MS/MS of a suitably digested product was fully consistent

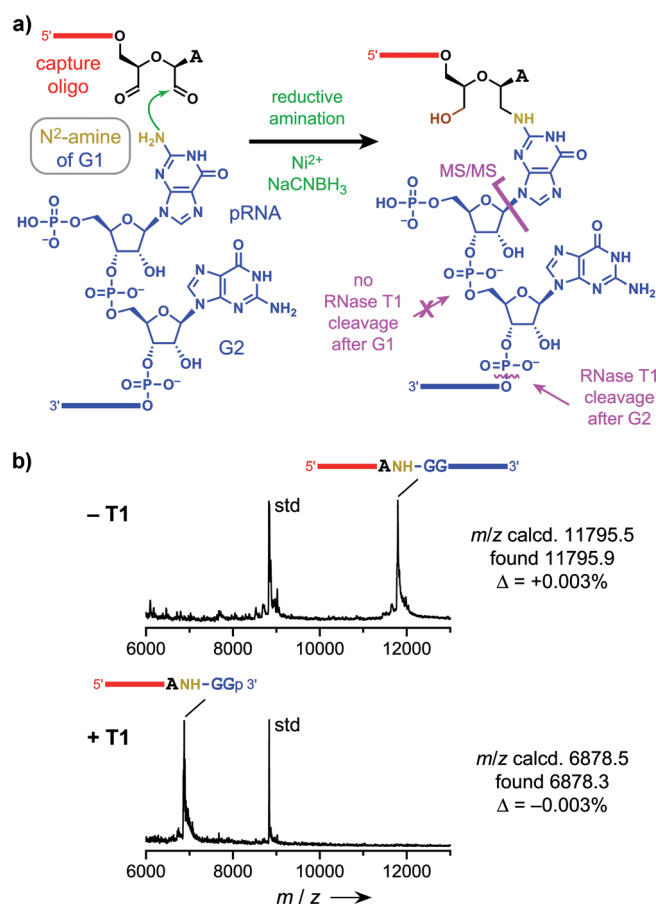


Figure 3. Proposed chemical structure of the reductive amination product, and support for this structure by mass spectrometry analysis. For each deoxyribozyme's product, the data do not distinguish which of the two aldehyde groups of the capture oligonucleotide has reacted. a) Chemical structures of reactants and reductive amination product. RNase T1 cannot cleave after G1 because of the modified guanine nucleobase, but RNase T1 cleavage after G2 is unaffected. The second aldehyde group of the product is shown as reduced to an alcohol on the basis of additional experimental data (see text). b) MALDI-TOF mass spectrometry of the 8QA124 product before and after RNase T1 digestion (see Table S1 in the Supporting Information for data for all studied deoxyribozymes). The data require that the capture oligonucleotide is attached to the RNA substrate at either G1 or G2 and also that G2 is available for RNase T1 digestion. The nucleotide connectivity illustrated in panel (a) is the only reasonable structure consistent with these data. MS/MS data (see Figure S6 in the Supporting Information) corroborate the assigned structure, especially through observation of fragmentation between the G1 ribose and G1 nucleobase as marked in panel (a).

with the assigned structure (see Figure S6 in the Supporting Information). In particular, a diagnostic MS/MS fragment was observed that corresponds to cleavage between the G1 ribose and the G1 nucleobase, as marked in Figure 3a.

In Figure 3a, the second aldehyde group of the capture oligonucleotide (which does not react with the guanine N²-amine through reductive amination) is depicted in reduced form as an alcohol. This structural assignment is based in part on three independent experiments which indicated that an aldehyde is not present at this position (data not shown). 1) The DNA-catalyzed reductive amination product is not

observed to be oxidized ($\text{CHO} \rightarrow \text{CO}_2\text{H}$) by sodium chlorite, NaClO_2 (Pinnick or Lindgren oxidation^[20]), which has been demonstrated to oxidize oligonucleotide-aldehyde substrates.^[21] In a positive control reaction, the capture oligonucleotide itself was observed to be oxidized by chlorite, with analysis by MALDI mass spectrometry. 2) The reductive amination product is not observed to react through a second reductive amination with several different amine-bearing oligonucleotides, under conditions that permit such reactivity in positive control reactions with a known aldehyde. 3) The reductive amination product is not observed to react with reagents such as biotin hydrazide, which in control assays reacts well with the capture oligonucleotide itself. This assignment of the second functional group as an alcohol rather than an aldehyde requires a reducing equivalent, which was presumably provided during the reaction by NaCNBH_3 . This reagent does not reduce aldehydes under near-neutral conditions, but the capture step during selection was performed at $\text{pH} \approx 5$, and NaCNBH_3 is known to be a synthetically useful reducing agent towards aldehydes and even ketones at pH values of 3–4,^[22] with examples at higher pH values such as 5.5.^[23] Therefore, the assigned aldehyde reduction by NaCNBH_3 is plausible, especially considering the additional presence of the Ni^{2+} cation as a Lewis acid to activate the aldehyde. Moreover, reduction of oligonucleotide-aldehyde substrates (see Figure S7 in the Supporting Information) by NaCNBH_3 under our incubation conditions is supported by direct experimental data (see Figure S8 in the Supporting Information). The success of the DNA-catalyzed reaction means that in the context of Figure 2b, DNA-catalyzed reductive amination is highly competitive with uncatalyzed aldehyde reduction.

The key finding of this study is that DNA is capable of catalyzing a reductive amination reaction, thereby broadening the repertoire of reactions known to be catalyzed by DNA (indeed by nucleic acids in general, as no such reactivity by ribozymes has been reported).^[1,2] It should be noted that DNA catalysis of reductive amination by deoxyribozymes as described here is quite distinct from DNA-templated reductive amination based on effective molarity,^[16,24] the key differences between DNA catalysis and DNA-templated synthesis were recently reviewed.^[2] In the presently reported DNA-catalyzed reaction, the electrophilic reaction partner, which is the dialdehyde formed by NaIO_4 oxidation of a 3'-terminal ribonucleotide, has considerable precedent for electrophilic reactivity.^[25] In contrast, although reaction of the guanine nucleobase at its N^2 -amine is experimentally precedented^[26] and has been proposed as a basis for codons to associate with amino acids during the origin of the genetic code,^[27] such nucleophilic reactivity is not extremely common.

An important mechanistic question is whether the new deoxyribozymes participate in catalysis of the imine formation step involving the N^2 -amine nucleophile, the reduction step involving NaCNBH_3 , or both of these reaction steps. The relatively low general nucleophilicity of the guanine N^2 -amine suggests that its nucleophilic attack into the dialdehyde-oligonucleotide would benefit strongly from DNA catalysis. For all seven of the new deoxyribozymes, NaCNBH_3 could be replaced with the alternative reducing agent dimethylamine-

borane or 2-picoline-borane,^[28] and substantial product yield was still observed (see Figure S9 in the Supporting Information). In contrast, Ni^{2+} cations could not be replaced with any of Mg^{2+} , Ca^{2+} , or Mn^{2+} cations (see Figure S10 in the Supporting Information). From these data, we infer that the deoxyribozymes likely catalyze the initial amination step (imine formation), whereas the subsequent reduction step is probably uncatalyzed. Moreover, we expect that the Ni^{2+} cation may serve as a Lewis acid both to foster DNA enzyme tertiary structure and to activate the aldehyde for DNA-catalyzed nucleophilic attack by the N^2 -amine. Ni^{2+} may furthermore enhance the reactivity of the imine during the uncatalyzed reduction step. Future work will involve more detailed investigation of the incubation conditions used for DNA-catalyzed reductive amination (e.g., change of pH, metal ion cofactor, or reducing agent), as well as exploration of other potential nucleophiles and electrophiles. Fully understanding the structures and mechanisms of the newly identified deoxyribozymes will require substantial further work, especially considering the many practical challenges associated with understanding these important features of other DNA catalysts.^[1,2,29]

Reductive amination is an important natural biosynthetic reaction^[12,13] that, through natural protein enzymes, has found utility in laboratory synthesis^[14] and in industry.^[15] The present work suggests that reductive amination by nucleic acid catalysts should be more broadly examined, perhaps in RNA World contexts by ribozymes. Others have commented upon the potential importance of reductive amination in such contexts,^[16,17] especially given the importance of nucleotide-based cofactors such as NAD(P)H in extant biochemistry. An artificial ribozyme that catalyzes alcohol dehydrogenation activity (not involving reductive amination) has been identified,^[30] as has a ribozyme for the reverse aldehyde reduction reaction.^[31]

One potential practical implication of the present findings relates to site-specific DNA and RNA modification. Because the nucleophile in the observed reductive amination reactions was the N^2 -amine of a guanine nucleobase, the findings suggest development of new deoxyribozymes that catalyze site-specific reactions at particular guanosine residues of DNA and RNA substrates. Site-specific internal modification of nucleic acids is a considerable challenge, and new approaches for this purpose would be valuable.^[32] In the current experiments, G1 was the only nucleotide of the RNA substrate that was not designed to engage in Watson–Crick base pairing (Figure 1b), which presumably suppressed nucleophilic reactivity of the nucleobase side chain of G2 or any other nucleobase within the RNA.

Finally, the outcome of this study illustrates a recurrent challenge of *in vitro* selection experiments: designing the selection strategy such that unanticipated reactions do not subvert the original intent.^[5,7,33] In this case, the nucleophilic reactivity of the guanine N^2 -amine was unexpected but is chemically plausible in the context of known examples.^[26] This reactivity is also interesting in the context of several biochemical and practical implications as discussed above. In our ongoing work, we are implementing suitably redesigned selection strategies to achieve our original goal of DNA-

catalyzed reactivity of side chains of untethered peptide substrates.

Experimental Section

Tripeptides were prepared by solid-phase synthesis using Fmoc Rink amide MBHA resin. DNA and RNA oligonucleotides prepared by solid-phase synthesis were obtained from Integrated DNA Technologies (Coralville, IA) or synthesized on an ABI 394 instrument using reagents from Glen Research. 5'-Triphosphorylated RNA oligonucleotides were prepared by in vitro transcription using synthetic DNA templates and T7 RNA polymerase.^[34] All oligonucleotides were purified by denaturing PAGE with running buffer 1×TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3) as described previously.^[19,35]

The random deoxyribozyme pool was 5'-CGAAGTCGC-CATCTCTTC-N₄₀-ATAGTGAGTCGTATTAAGCTGATCCTGATGG-3'. PCR primers were 5'-CGAAGTCGCCATCTCTTC-3' (5'-phosphorylated to allow ligation using T4 RNA ligase to the 3'-terminus of the 5'-triphosphorylated RNA substrate) and 5'-(AAC)_XCCATCAGGATCAGCTTAATACGACTCACTAT-3' (where X is Glen Spacer 18 to stop Taq polymerase). The RNA substrate was 5'-GGAAGAGAUGGC-GACGG-3', prepared by in vitro transcription using T7 RNA polymerase (for 5'-triphosphate) or by solid-phase synthesis (for 5'-monophosphate or 5'-hydroxy). The helper oligonucleotide was 5'-(AAC)_YGGATAATACGACTCACTAT-3', where the 5'-extension was included so that any deoxyribozymes that catalyze attack of the helper 3'-hydroxyl into the RNA 5'-triphosphate would be shifted very high on PAGE and therefore not selected. The 3'-dialdehyde-terminated capture oligonucleotide was prepared from 5'-GGATAATACGACTCACTAT (rA)-3' by oxidation of 20–5000 pmol of oligonucleotide in 20–50 μL volume of 100 mM HEPES, pH 7.5, and 10 mM NaIO₄ at room temperature for 1 h followed by ethanol precipitation. Equivalent reactivity was observed when the capture oligonucleotide was additionally purified by Amicon filtration (3 kDa cutoff; data not shown). Oxidation of the capture oligonucleotide to form the 3'-dialdehyde, already very well-precedented,^[25] was further validated by mass spectrometry (see Figure S7 in the Supporting Information).

The in vitro selection and cloning procedures were performed as described previously,^[11] with modifications as described in the Supporting Information. Individual deoxyribozymes were prepared by solid-phase synthesis and assayed using 3'-³²P-radiolabeled RNA substrates, which were prepared from the unlabeled RNA using 5'-³²P-pCp and T4 RNA ligase.^[11] The general assay procedure for each deoxyribozyme was as follows. A 10 μL sample containing 1 pmol of 3'-³²P-radiolabeled RNA substrate, 10 pmol of deoxyribozyme, and 20 pmol of capture oligonucleotide was annealed in 5 mM HEPES, pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95°C for 1 min and cooling on ice for 5 min. The reductive amination reaction was initiated by bringing the sample to 20 μL total volume containing 100 mM NaOAc, pH 5.2, 50 mM NiCl₂, and 10 mM NaCNBH₃ and incubating at 37°C. The NaCNBH₃ was added from a 200 mM aqueous stock solution. At appropriate times, 2 μL aliquots were quenched with 6 μL of stop solution (80% formamide, 1×TBE [89 mM each Tris and boric acid, 2 mM EDTA, pH 8.3], 50 mM EDTA, and 0.025% each bromophenol blue and xylene cyanol). Before PAGE, to each sample was added 100 pmol of a decoy oligonucleotide, which was a 60-mer complementary to the 40 nt catalytic region of the deoxyribozyme (to displace deoxyribozyme from substrate and product). Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of *k*_{obs} were obtained by fitting the yield versus time data directly to first-order kinetics.

Samples for MALDI-TOF mass spectrometry (Figure 3b) were prepared using a modified version of the above assay procedure (40 μL annealing volume, 80 μL final volume, 1 nmol 5'-monophosphorylated RNA substrate, 1.2 nmol deoxyribozyme, 1.5 nmol capture oligonucleotide, 37°C, 14 h, ethanol precipitation, and purifica-

tion by 20% PAGE). RNase T1 digestion was performed as described previously.^[11] See the Supporting Information for MS/MS sample preparation.

Received: July 15, 2011

Revised: September 22, 2011

Published online: October 12, 2011

Keywords: deoxyribozymes · DNA · enzyme catalysis · reductive amination

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