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Enhanced Deoxyribozyme-Catalyzed RNA Ligation in the Presence of Organic Cosolvents

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Abstract

Deoxyribozyme and aptamer selections are typically conducted in aqueous buffer solutions. Using nonaqueous cosolvents in selection experiments will help expand the activity of deoxyribozymes with non-oligonucleotide substrates and will allow identification of new aptamers for nonprotein targets. We undertook *in vitro* selections utilizing a small amount of methanol in the reaction to keep the herbicides alachlor and atrazine in solution with the goal of identifying deoxyribozymes that require these herbicides for activity. The resulting deoxyribozymes successfully catalyze RNA ligation, but do not require alachlor or atrazine. Surprisingly, some of these deoxyribozymes displayed better catalytic activity in the presence of methanol over just aqueous buffer. We investigated several organic cosolvents to see if this enhancement was limited to methanol and found that other cosolvents, including ethanol, DMSO, and DMF, supported activity; in some cases, greater enhancement was observed. On the basis of these results, we tested two other previously identified RNA-ligating deoxyribozymes to assess their tolerance of cosolvents and determined that different deoxyribozymes showed different responses to the cosolvents. Our results demonstrate that deoxyribozymes can tolerate and, in some cases, display enhanced activity in alternative solvent conditions. These findings will facilitate the development of responsive deoxyribozyme systems utilizing components with limited water solubility.

Keywords

deoxyribozyme; DNAzyme; in vitro selection; RNA ligation

INTRODUCTION

Deoxyribozymes, or DNAzymes, have been identified via *in vitro* selection experiments by their ability to catalyze a desired reaction.^{1,2} To date, most reported deoxyribozymes were selected in aqueous buffer solutions because the reactants were fully water-soluble. The Diels-Alderase deoxyribozyme, like its ribozyme counterpart,^{3,4} is active with small amounts of DMSO or ethanol, which keeps the substrates soluble.⁵ Recent work with peptide-containing substrates included a small amount of DMF in the selection reaction.⁶ The peroxidase deoxyribozyme also utilizes a small amount of DMSO to form the active complex between hemin and the DNA,⁷ but after it is formed, this complex displays activity in the absence of this cosolvent.⁸ In these examples, an organic cosolvent was necessary to maintain the solubility or stability of one of the reaction components, but the effect of the cosolvent on DNA catalysis itself was not or could not be investigated. A recent study investigated the formation and peroxidase activity of G-quadruplex sequences in organic

solvents; however, the oligonucleotides required polyethylene glycol (PEG) modification for solubility.⁹ It is unclear how the addition of large PEG units would affect other deoxyribozymes that do not have stable G-quadruplexes in their structures.

To expand the catalytic abilities of deoxyribozymes, new substrates, including those with limited water solubility, need to be explored. In addition, the development of aptazymes, oligonucleotide sequences that use ligand binding to aptamer domains to regulate nucleic acid catalysis,¹⁰ may also require the use of organic cosolvents for improved target solubility or to mimic complex sample media. In our efforts to identify new DNA aptazymes whose catalytic activities are dependent on the binding of herbicide targets with low water solubility, we conducted *in vitro* selection experiments using methanol as a cosolvent. The resulting RNA-ligating deoxyribozymes did not need the herbicides for catalysis, but further characterization determined that several of these sequences displayed enhanced catalytic activity in the presence of methanol as compared to aqueous buffer. On the basis of these results, we investigated if other alcohols and cosolvents could support and possibly enhance deoxyribozyme activity, not only for our new deoxyribozymes, but also for two previously identified RNA-ligating deoxyribozymes (Figure 1).

MATERIALS AND METHODS

General Experimental Considerations

DNA and RNA oligonucleotides were prepared by solid-phase synthesis at IDT (Coralville, IA). RNA transcripts were prepared by *in vitro* transcription using T7 RNA polymerase and a double-stranded DNA template prepared by annealing two synthetic DNA oligonucleotides.^{11,12} Oligonucleotides and RNA transcripts were purified by denaturing PAGE using 1X TBE running buffer (89 mM each Tris and boric acid, 2 mM EDTA, pH 8.3). Samples were eluted from the gel by the crush-and-soak method in 10 mM Tris pH 8.0, 1 mM EDTA, 300 mM NaCl (TEN) buffer, recovered by ethanol precipitation, and quantified by UV absorbance. The sequences of the RNA substrates used for *in vitro* selection were 5'-GGAACUGC-GAUCUAGUGA-3' (L RNA) and 5'-pppGACUGACUCGU-GAUCGGA-3' (R RNA), where ppp represents the triphosphate installed during *in vitro* transcription. The RNA substrates used with 10DM24 were 5'-GGAUAAUACGACUCACUAUA-3' (L RNA) and 5'-pppGGAAGAGAUGGCGACGG-3' (R RNA). The RNA substrates used with 9DB1 were 5'-GGAAGUCUCAUGUACUA-3' (L RNA) and 5'-pppGAUGUUCUAGCGCCGGA-3' (R RNA).

In Vitro Selections—*In vitro* selections were undertaken to identify RNA-ligating deoxyribozymes that depend on a target herbicide for activity. The selection procedure is provided in detail in the Supporting Information and was based on previously reported efforts.¹³ The selection conducted with alachlor as the target was designated selection Q and the selection with atrazine was designated selection R. After 10 rounds, ligation activity was present in both selection efforts. DNA sequences from rounds 9 and 10 of both selection efforts were amplified using PCR primers that generate a double-stranded product. The products were purified by agarose gel and cloned using the TOPO® TA cloning kit (Invitrogen, Carlsbad, CA). Individual colonies were cultured and the resulting plasmids were isolated via miniprep (IBI Scientific, Peosta, IA). Plasmids were then sequenced by ACGT. (Wheeling, IL). Unique DNA sequences were made by solid-phase synthesis by IDT for testing.

Kinetic Studies—9R20 and Related Deoxyribozymes

The ligation abilities of the newly identified deoxyribozymes were tested with the RNA substrates used in selection. The L RNA substrate was 5'-end radiolabeled with γ -³²P-ATP

and T4 polynucleotide kinase. All assays were conducted under conditions used during selection, except the R RNA was no longer covalently linked to the DNA enzyme, resulting in the previously described trimolecular format.^{14,15} The ratio of 5' end radiolabeled L substrate (*L) to deoxyribozyme (E) to R substrate (R) was 1:5:10, where the concentration of deoxyribozyme was 0.5 μ M. This ratio was used to ensure all of *L is bound to a deoxyribozyme and that all *L-E complexes are bound to R. Other ratios that maintain *L < E < R could also be used. The appropriate oligonucleotides were annealed by heating at 95°C for 3 min, followed by incubation on ice for 5 min in 5 mM HEPES pH 7.5, 15 mM NaCl, and 0.1 mM EDTA. The reactions were initiated by addition of the reaction buffer and the target herbicide. Final reaction conditions were 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, 40 mM MgCl₂, 20 mM MnCl₂, 1 mM alachlor (selection Q) or atrazine (selection R), and 10% methanol (from the addition of alachlor or atrazine) in 20 μ l at 22°C. Deoxyribozymes were also assayed in the absence of the herbicide target by omitting the herbicide but maintaining 10% methanol in the reaction, as well as by omitting the methanol (aqueous buffer conditions). For cosolvent studies, 10% of the appropriate cosolvent was included in place of the methanol as indicated in the figures. Concentration studies were also conducted by changing the final % composition with regard to the cosolvent. In all these cases, the other reaction components (buffer, ions, and oligonucleotides) were maintained in the same concentrations as used in assays with the herbicide. At appropriate time points, 1.5 μ l was removed from each reaction and quenched into 8 μ l stop solution containing 80% formamide, 1 $\times$ TB [89 mM each Tris and boric acid, pH 8.3], 50 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanole. Samples were separated by 20% denaturing PAGE and imaged using a PhosphorImager (GE Health-care, Piscataway, NJ). Gel images were analyzed using Quantity One (Bio-Rad, Hercules, CA) and the data were fit using KaleidaGraph (Synergy Software, Reading, PA) to first-order kinetics using $\text{yield} = Y \cdot (1 - e^{-kt})$, where $k = k_{\text{obs}}$ and Y = final yield. Deoxyribozymes that did not show ligation activity in the initial screen with 10% methanol or the herbicide target were assayed by enzymatically ligating the R RNA substrate to a ³²P-labeled deoxyribozyme generated via PCR with α -³²P-dCTP, as was done in selection. The approach reintroduces the loop region and the covalent linkage between the R RNA substrate and the deoxyribozyme. The resulting R RNA-deoxyribozyme complex was then incubated with unlabeled L RNA under the reaction conditions described above and products were separated by 8% denaturing PAGE.

Kinetic Studies—10DM24 and 9DB1

The ligation abilities of 10DM24,¹⁶ 5'-CCGTCGCCATCTCCCG-TAGGTGAAGGGCGTGAGGGTTCCATTCCCGTATTATCC-3', and 9DB1,¹⁷ 5'-GCTAGAACATGGATCATACGGTCGGAGGGGTTTGC-CGTGAACATTCTTCAAGTACATGAG-3', were tested using the short RNA sequences originally used for selection as listed above. The L RNA substrate was 5' end radiolabeled with γ -³²P-ATP and T4 polynucleotide kinase. All assays were conducted under conditions similar to those previously reported for studies of 10DM24^{14–16} and 9DB1.^{17–19} Prior to initiating the reaction, appropriate oligonucleotides were annealed in 5 mM HEPES pH 7.5, 15 mM NaCl, and 0.1 mM EDTA by heating at 95°C for 3 min and cooling on ice for 5 min. The reaction buffer, MgCl₂, and appropriate cosolvent were then added to initiate the reactions. Final incubation conditions were 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, and cosolvent at 22°C as indicated in figure legends. For Mg²⁺ concentration studies, the concentration of MgCl₂ was varied from 1 to 40 mM as indicated in the figure legends. At appropriate time points, 1.5 μ l was removed from each reaction and quenched into 8 μ l stop solution. Samples were separated by 20% denaturing PAGE. The resulting gels were imaged and analyzed as described above.

RESULTS AND DISCUSSION

In Vitro Selection of RNA-Ligating Aptazymes and the Emergence of Alcohol-Enhanced Deoxyribozymes

As part of our ongoing work to identify and develop deoxyribozymes that function as aptazymes in the presence of a small-molecule target,¹⁴ we initiated a set of *in vitro* selections for RNA-ligating DNA aptazymes that are reliant on either alachlor (designated selection Q) or atrazine (designated selection R) for activity. These two herbicides have limited water solubility, which restricts the concentration of aqueous stock solutions that can be made for each of these molecules. To allow for concentrated 10× stock solutions of 10 mM, the compounds were dissolved in methanol. Use of this stock introduced 10% methanol into our final selection reaction volumes. For consistency, this amount of methanol was maintained for negative selection steps in which the herbicides were not introduced into the reactions. After 10 rounds of selection, at least 20% ligation activity was present in both efforts (Supporting Information Figure S1). The selection products were cloned and sequenced. From the two efforts taken together, a total of 12 unique sequences were identified (Supporting Information Table S1). Our characterization studies of the newly identified catalytic DNA sequences revealed that the deoxyribozymes did not function as aptazymes dependent on the target herbicide for activity. The deoxyribozymes were capable of RNA ligation, but were equally active in 10% methanol with or without 1 mM of either alachlor or atrazine (Supporting Information Table S2). The deoxyribozymes were also rather slow, requiring overnight incubation for maximum yields despite a 2 h selection reaction time.

Through the course of our studies, we noticed a trend for some of our deoxyribozymes. While the average ligation activity difference between reactions where methanol was included and reactions where methanol was omitted was not significant (Supporting Information Table S3), some of the deoxyribozymes consistently showed better ligation activity with methanol in independent trials where reactions with and without methanol were conducted side-by-side (data not shown). This result was initially unexpected, but methanol was present during the selection, so methanol dependence could readily emerge for the active sequences. On the basis of this observation, we set out to characterize the apparent dependence on methanol as a cosolvent. We focused our efforts on deoxyribozyme 9R20 because it consistently produced about double the amount of ligated product with methanol versus aqueous buffer in independent trials. When 10% methanol was included in the ligation reaction, 9R20 produced on average 42% ligated product as compared to just 21% in aqueous buffer after overnight incubation (Figure 2A). Although included simply because it was the solvent for the herbicide targets in selection, methanol could be playing an additional role in the ligation reaction. Methanol is known to influence nucleic acid structure^{20,21} and may have become integral to the formation of the active conformation of the deoxyribozymes. Previous work investigating the Mg²⁺-mediated folding of RNA demonstrated that methanol stabilizes tertiary structure, but not secondary structure.²² For our deoxyribozyme, the absence of methanol may be preventing the formation of a weak tertiary structure.

Cosolvent Enhancement of 9R20 Activity—On the basis of the results with methanol, we tested other alcohols (isoamyl alcohol and ethanol), acetone, *N,N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) to see if these other cosolvents were able to enhance the ligation activity of 9R20. We anticipated that ethanol would also enhance ligation activity because ethanol is known to affect nucleic acid structure,²³ similarly to methanol. DMF is commonly used with peptide nucleic acids (PNAs) and lowers the stability of DNA duplexes,²⁴ while DMSO is used as an additive in PCR and DNA

sequencing reactions because it disrupts base-pairing and secondary structure.²⁵ Such additives may prevent the formation of inactive deoxyribozyme structures formed by alternative base-pairing within the enzyme region. DMF and DMSO are also commonly used as cosolvents for labeling or crosslinking reactions involving biomolecules and NHS esters, which have limited water solubility. A potential unwanted effect in our study is that the addition of these cosolvents will reduce the base-pairing stability between the RNA substrates and the deoxyribozyme, leading to substrate dissociation and reduced ligation activity. However, the disruption of base-pairing between the deoxyribozyme and the ligated product could facilitate product release.

For the cosolvent tests, we included 10% of the appropriate cosolvent in the final reaction volume. Ten percent ethanol produced the greatest activity enhancement, yielding 56% ligated product after overnight incubation (Figure 2A), more than 2.5-fold higher than without ethanol. Other solvents showed more modest yield enhancements, with isoamyl alcohol providing almost no yield improvement (Table I). When other deoxyribozymes from the selections that showed enhanced activity with methanol were tested with these different solvents, ethanol, methanol, and in some cases acetone, consistently produced the highest yields (Supporting Information Figure S2).

Because 10% ethanol led to 2.6-fold higher ligated product for 9R20 as compared to aqueous buffer and a 1.4-fold increase when compared to 10% methanol, we investigated the ethanol concentration dependence of the ligation reaction by varying the concentration from 0 to 40% (Figure 2B). The highest ligation yields were obtained with 15–20% ethanol, with a 3.3-fold increase to 70% ligated product obtained after overnight incubation as compared to aqueous buffer (Table I). There was a sharp decrease in activity when the ethanol concentration was increased to 25% and activity was completely lost at 30% ethanol (Figure 2B). 20% ethanol has been used in studies on RNA folding²³ and may represent an optimum concentration for stabilizing nucleic acid structure. At higher ethanol concentrations, nucleic acid precipitation could begin to interfere with the ligation reaction, although other studies investigating DNA denaturation have used higher concentrations.^{26,27}

The Effect of Cosolvents on 9R20's Divalent Metal Ion Requirements—Early studies with RNase P demonstrated that ethanol can support high levels of catalysis with suboptimal Mg^{2+} concentrations.²⁸ A similar effect for deoxyribozymes could allow for lower concentrations of divalent metals, which may protect RNA substrates from metal-induced degradation. We determined if the presence of either methanol or ethanol alters the divalent metal ion requirement for catalysis by 9R20.

In our selections, we utilized a mixture of 40 mM Mg^{2+} and 20 mM Mn^{2+} . Mg^{2+} is commonly used for deoxyribozyme selections, but mixtures of divalent metals have been used successfully.^{5,29–31} Mn^{2+} also supports deoxyribozyme-catalyzed RNA and DNA ligation^{32,33} and can replace Mg^{2+} for some deoxyribozymes.^{15,34} We determined if just one of the metals could support catalysis or if both were required. We also included either 10% methanol or 20% ethanol to determine if these cosolvents could support catalysis with either Mg^{2+} or Mn^{2+} alone.

Figure 3 shows how 9R20 responds when given only Mg^{2+} or Mn^{2+} in the presence and absence of methanol or ethanol. When assayed in aqueous buffer or 10% methanol, 9R20 works better with Mn^{2+} alone than Mg^{2+} alone, with 33% ligation yield from reactions with 20 mM Mn^{2+} in 10% methanol. However, the same result was not observed when 20% ethanol was used as the cosolvent. In fact, we observed reaction inhibition with 20% ethanol and 20 mM Mn^{2+} . For 40 mM Mg^{2+} , essentially no ligation activity was observed in aqueous buffer or 10% methanol. A small amount of ligation is recovered with 20% ethanol,

but the resulting ~12% yield is still less than the activity with Mn^{2+} alone in 10% methanol. These results are interesting considering that 20% ethanol was our best cosolvent when both metal ions were present, yielding ~70% ligated product. While several different models could potentially account for our observations, one possible explanation is that Mg^{2+} is necessary to stabilize an active deoxyribozyme structure that places Mn^{2+} in a catalytic role. In this model, the active structure is enhanced or favored in the presence of ethanol when both metals are present, but not if only Mn^{2+} is available. If only Mg^{2+} is available, ethanol stabilizes the active conformation of the deoxyribozyme in such a way that Mg^{2+} can take over the catalytic role, albeit with less efficiency. Methanol may be better than ethanol at stabilizing the active deoxyribozyme structure when no Mg^{2+} is available, which allows for catalysis when only Mn^{2+} is present.

Cosolvent Effects on Other RNA-Ligating Deoxyribozymes—The results with our new deoxyribozymes inspired us to investigate other RNA-ligating deoxyribozymes to determine if they are also affected by organic cosolvents. 9R20 emerged from selections that included methanol in the selection reaction, so observing improved activity with methanol and related cosolvents is not completely surprising. By investigating how other deoxyribozymes respond to these cosolvents, we can assess if our observed enhancements for 9R20 are a result of the presence of the cosolvent in the selection or if they represent a more general effect. These results will also provide useful information about the activities of deoxyribozymes under nonstandard conditions. We chose two previously identified deoxyribozymes, 10DM24 and 9DB1, for these cosolvent studies. 9R20 catalyzes ligation of two RNA substrates to form non-native, 2'–5' linear linkages (Supporting Information Figure S3). 10DM24 and 9DB1 both catalyze RNA ligation reactions, but form different linkages and display different reaction rates than 9R20, as described below.

Studies of the 10DM24 Deoxyribozyme—The 10DM24 deoxyribozyme catalyzes an RNA-branching reaction between an internal 2'-OH of one RNA substrate and the 5' triphosphate of a second RNA substrate.^{15,35} We have previously demonstrated that the 10DM24 deoxyribozyme is amenable to aptazyme conversion¹⁴ and could be useful in other types of applications, so we were interested in how 10DM24 would respond to the presence of different additives. We tested 10DM24 using the same cosolvents studied with 9R20 (Figure 4A). 10DM24 is virtually unaffected by the addition of 10% of the tested cosolvents, indicating the formation of a stable, active configuration regardless of the solvent conditions (Table II). Surprisingly, 10DM24 maintained significant ligation activity with 40% ethanol in the reaction and activity was not completely abolished even with 60% ethanol present (Figure 4B). The reason for this unexpected stability is unknown but our results indicate a very stable catalytic conformation.

In previous work with 10DM24, ligation yields were reduced when nucleotide triphosphates, such as ATP and GTP, were added to the reaction.^{14,36} These decreased reaction yields were presumably due to Mg^{2+} chelation and indicated that the deoxyribozyme does not tolerate decreases in Mg^{2+} concentration. We investigated if the addition of ethanol could compensate for lower Mg^{2+} concentrations (Figure 5). We tested concentrations ranging from 1 to 40 mM Mg^{2+} in the presence and absence of 20% ethanol. 10DM24 maintained high ligation yields in 2 h with as little as 5 mM Mg^{2+} (Table III). Decreasing the Mg^{2+} concentration to 1 mM led to a significant drop in ligation yields to 23% in 2 h. The addition of 20% ethanol had very little effect on ligation yield with decreasing Mg^{2+} until the concentration was dropped to 1 mM. At 1 mM Mg^{2+} with 20% ethanol, 10DM24 maintained substantial activity, with 65% product formed in 2 h. These results indicate that ethanol can rescue 10DM24 ligation activity under low Mg^{2+} conditions.

Overall, 10DM24 readily tolerates the addition of cosolvents and maintained its catalytic activity at extremely high concentrations of ethanol. These results indicate that 10DM24 does not readily misfold and its active form is not easily disrupted in a way that would decrease catalytic activity. While previous results had indicated decreases in Mg^{2+} concentrations were not well-tolerated,^{14,36} our current studies show that 10DM24 is still very functional at lower Mg^{2+} concentrations and the addition of 20% ethanol allows ligation with useful yields with just 1 mM Mg^{2+} . 10DM24 has previously been used with large RNAs in the DECAL labeling approach.¹⁵ Lower Mg^{2+} concentrations would be particularly useful for these types of reactions using long RNA substrates, which are susceptible to metal-catalyzed, nonspecific degradation. Also, we have previously shown that 10DM24 is amenable to aptazyme conversion,¹⁴ and our studies here demonstrate that 10DM24 would be a possible candidate for aptazymes involving aptamers identified under conditions involving cosolvents because its activity appears to be unaffected by cosolvents.

Studies of the 9DB1 Deoxyribozyme—9DB1 catalyzes the formation of a 3′–5′ native linear linkage between two RNA substrates.^{17–19} While the catalytic structure has been minimized,¹⁸ we maintained the full enzyme region for our experiments. During initial studies, we found that simply shortening the binding arms from 16 to 10 bp each improved catalytic activity under our conditions (data not shown), so we used this shorter binding arm version in our studies. We also allowed the reactions to proceed overnight (~24 h) to obtain maximum yields. Once again, we used the same cosolvents as used with 9R20 to assess the ligation abilities of 9DB1. Unlike 10DM24, we did see small effects on the ligation activity of 9DB1, mainly in terms of reaction rate (Figure 6A). Methanol, ethanol, and DMSO improved the reaction rates and produced slightly higher yields than the other cosolvents (Table IV). Aqueous buffer conditions produced the lowest yields. We also tested 9DB1 with different amounts of ethanol ranging from 0% to 60% to determine the optimum amount of ethanol to include in the reactions. As seen in Figure 6B, 5–30% ethanol supported high ligation yields (>90%) after overnight incubation, but the reaction rates varied. The reaction was fastest with 15–30% ethanol (Table IV). Inclusion of more ethanol in the reaction led to decreased product yields, with complete loss of activity in 60% ethanol. For 9DB1, the inclusion of ~20% ethanol improves the ligation activity and allows >90% product in just 3 h, which improves the practical usefulness of this deoxyribozyme.

In aqueous buffer, 9DB1 was much more sensitive to decreases in Mg^{2+} concentration than was 10DM24 (Figure 7). Decreasing the Mg^{2+} concentration from 40 to 20 mM led to a drop from 71% to just 28% ligation and further decreases resulted in <2% ligation (Table III). The inclusion of 20% ethanol rescued activity and concentrations as low as 5 mM Mg^{2+} was tolerated with very little loss in activity (Figure 7 and Table III).

9DB1, like 9R20, showed enhanced activity in the presences of certain cosolvents. This result was somewhat unexpected as 9DB1 had not been subjected to cosolvent conditions during its selection.¹⁷ Previous work has shown that regions of the originally selected enzyme region are not necessary for catalysis.¹⁹ The improved activity in methanol, ethanol, and DMSO indicates that 9DB1 is susceptible to misfolding or an interfering secondary structure, possibly involving these unnecessary nucleotides, that is disrupted by these cosolvents. Similar to 10DM24, 9DB1 retains activity at surprisingly high concentrations of ethanol, indicating that the active structure, once properly formed, is stable under these conditions. The addition of ethanol also had a dramatic effect on the Mg^{2+} concentration dependence, with 9DB1 maintaining useful activity with 5 mM Mg^{2+} in 20% ethanol. The addition of ethanol allows for lower Mg^{2+} concentrations, which as discussed for 10DM24, could protect the RNA substrates and products from metal-catalyzed degradation. 9DB1 has not been subjected to aptazyme conversion, but our results presented here and previous

studies¹⁹ could lead to the development of aptazyme systems similar to those possible with 10DM24.¹⁴

CONCLUSIONS

The stability of DNA under a variety of conditions has led to much interest in incorporating DNA into sensors and nano-devices.^{2,37} Functional nucleic acids, such as aptamers and deoxyribozymes, can add enhanced capabilities to these systems. Learning how deoxyribozymes function beyond standard aqueous conditions will aid the development of responsive systems, such as aptazymes, that require components with limited water solubility.

While a few deoxyribozymes have been selected in the presence of organic cosolvents because of substrate solubility considerations, the RNA-ligating deoxyribozymes that emerged from our selection efforts proved to be an ideal system for investigating the effect of cosolvents on deoxyribozyme catalysis. We were also able to look at deoxyribozymes that catalyze similar ligation reactions, but had never been exposed to cosolvents in selection. Our results demonstrate that deoxyribozymes can be quite tolerant of organic cosolvents in the reaction, regardless of whether that cosolvent was present during selection. The majority of our tested cosolvents are known to have an effect on nucleic acid structure. One possible reason for the observed activity enhancement, especially for 9R20 and 9DB1, is dehydration of the nucleic acids by the cosolvent, which can stabilize certain structures and may influence the active deoxyribozyme conformation. A dehydrating environment may also prevent the formation of alternative, nonactive deoxyribozyme conformations, similar to the effects observed for human telomere sequence-derived G-quadruplex DNA in water-free solvents.³⁸ The cosolvents could also be acting as crowding agents that improve the association between the deoxyribozyme and its substrates, increasing the number of active complexes. The increase in active complexes can improve the reaction rate, as was shown for the hammerhead ribozyme when partitioned in an aqueous two-phase system using crowding agents.³⁹ Our results indicate that the cosolvents do not necessarily need to be present during a selection to later provide activity enhancement. However, we were unable to observe any specific trends concerning the ability of a particular cosolvent to improve the yield or enhance the rate for our tested deoxyribozymes. The specific response cannot be readily predicted for any given deoxyribozyme, which highlights the need to assess deoxyribozyme activities when reaction conditions are altered from the selection conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Additional Supporting Information may be found in the online version of this article.

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FIGURE 1.

RNA-ligating deoxyribozymes tested in this study. Deoxyribozymes are shown in gray, the L RNA substrates are shown in red, and the R RNA substrates are shown in blue. 9R20 catalyzes ligation between the terminal 2' OH of the L RNA and the 5' triphosphate of the R RNA, resulting in a non-native, linear linkage. 9DB1 catalyzes ligation between the 3' OH of the L RNA and the 5' triphosphate of the R RNA, resulting in a native, linear linkage.¹⁷ 10DM24 catalyzes ligation between an internal 2' OH of the L RNA and the 5' triphosphate of the R RNA, resulting in a branched RNA linkage.¹⁶ The thin gray lines indicate an additional base-pairing interaction between the 10DM24 deoxyribozyme and the R RNA.

FIGURE 2.

Cosolvent studies of deoxyribozyme 9R20. (A) Testing 9R20 with a series of cosolvents. Assays were conducted using 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, 40 mM MgCl_2 , 20 mM MnCl_2 , and 10% of the tested cosolvent at 22°C. The tested cosolvents were water (gray circles), methanol (black squares), ethanol (blue wedges), isoamyl alcohol (green diamonds), acetone (purple triangles), DMSO (red triangles), and DMF (brown wedges). (B) Ethanol concentration studies. Assays were conducted using 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, 40 mM MgCl_2 , 20 mM MnCl_2 , and ethanol concentrations ranging from 0 to 40% at 22°C. The tested concentrations were 0% (gray circles), 5% (black squares), 10% (blue diamonds), 15% (green triangles), 20% (purple triangles), 25% (red wedges), 30% (brown wedges), 35% (orange crosses), and 40% (open black triangles). Time points were at 0, 1, 3, 6, and overnight (>18 h). Data were fit to first-order kinetics as described in the Experimental section. Error bars for data points with three or more trials represent the standard deviation, while error bars for data points with two trials represent the data range.

FIGURE 3.

Metal ion specificity for deoxyribozyme 9R20. Representative gel of 9R20 activity tests under single divalent metal ion conditions. Assays were conducted using 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, and either 20 mM MnCl₂ or 40 mM MgCl₂ at 22°C in aqueous buffer, 10% methanol, or 20% ethanol. Time points were at 0, 1, 3, 6, and overnight (>18 h). Unreacted ³²P-labeled L RNA was separated from the ligated product by 20% denaturing PAGE. Final reaction yields for two independent trials were 16%, 33%, and 8% for the 20 mM Mn²⁺ reactions with aqueous buffer, 10% methanol, and 20% ethanol, respectively and were <0.1, 1, and 12% for the 40 mM Mg²⁺ reactions with aqueous buffer, 10% methanol, and 20% ethanol, respectively.

FIGURE 4.

Cosolvent studies for 10DM24 deoxyribozyme. (A) Testing 10DM24 with a series of cosolvents. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, and 10% of the tested cosolvent at 22°C. The tested cosolvents were water (gray circles), methanol (black squares), ethanol (blue wedges), isoamyl alcohol (green diamonds), acetone (purple triangles), DMSO (red triangles), and DMF (brown wedges). (B) Ethanol concentration studies. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, and ethanol concentrations ranging from 0 to 60% at 22°C. The tested concentrations were 0% (gray circles), 5% (black squares), 10% (blue diamonds), 20% (green triangles), 30% (purple triangles), 40% (red wedges), 50% (brown wedges), 55% (orange crosses), and 60% (open black triangles). Time points were at 0, 5, 15, 30, 60, and 120 min. Data were fit to first-order kinetics as described in the Experimental section. Error bars for data points with three or more trials represent the standard deviation, while error bars for data points with two trials represent the data range.

FIGURE 5.

Metal concentration studies for 10DM24 deoxyribozyme. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, and Mg^{2+} concentrations ranging from 1 to 40 mM at 22°C in the presence (solid lines) and absence (dashed lines) of 20% ethanol. The tested concentrations were 40 mM (black circles), 20 mM (blue squares), 10 mM (red diamonds), 5 mM (green triangles), and 1 mM (purple triangles). Time points were at 0, 5, 15, 30, 60, and 120 min. Data were fit to first-order kinetics as described in the Experimental section. Error bars for data points with three or more trials represent the standard deviation, while error bars for data points with two trials represent the data range.

FIGURE 6.

Cosolvent studies for 9DB1 deoxyribozyme. (A) Testing 9DB1 with a series of cosolvents. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, and 10% of the tested cosolvent at 22°C. The tested cosolvents were water (gray circles), methanol (black squares), ethanol (blue wedges), isoamyl alcohol (green diamonds), acetone (purple triangles), DMSO (red triangles), and DMF (brown wedges). (B) Ethanol concentration studies. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, and ethanol concentrations ranging from 0 to 60% at 22°C. The tested concentrations were 0% (gray circles), 5% (black squares), 10% (blue diamonds), 15% (green triangles), 20% (purple triangles), 30% (red wedges), 40% (brown wedges), 50% (orange crosses), and 60% (open black triangles). Time points were at 0, 1, 3, 5, 8, and overnight (~24 h). Data were fit to first-order kinetics as described in the Experimental section. Error bars for data points with three or more trials represent the standard deviation, while error bars for data points with two trials represent the data range.

FIGURE 7.

Metal concentration studies for 9DB1 deoxyribozyme. Assays were conducted using 50 mM CHES pH 9.0, 150 mM NaCl, 2 mM KCl, and Mg^{2+} concentrations ranging from 1 to 40 mM at 22°C in the presence (solid lines) and absence (dashed lines) of 20% ethanol. The tested concentrations were 40 mM (black circles), 20 mM (blue squares), 10 mM (red diamonds), 5 mM (green triangles), and 1 mM (purple triangles). Time points were at 0, 1, 3, 5, 8, and overnight (~24 h). Data were fit to first-order kinetics as described in the Experimental section. Error bars for data points with three or more trials represent the standard deviation, while error bars for data points with two trials represent the data range.

Table IYields and Observed Rate Constants for 9R20^a

Cosolvent Conditions	% Yield	Fold Increase in Yield ^b	<i>k</i> _{obs}
Aqueous buffer	21	–	0.070
10% methanol	42	2	0.092
10% isoamyl alcohol	25	1.2	0.058
10% acetone	36	1.7	0.104
10% DMSO	45	2.1	0.063
10% DMF	33	1.6	0.045
Ethanol concentration			
5% ethanol	49	2.3	0.10
10% ethanol	56	2.6	0.102
15% ethanol	70	3.3	0.128
20% ethanol	70	3.3	0.133
25% ethanol	16	–	0.09
30% ethanol	<1	–	–
35% ethanol	<1	–	–
40% ethanol	<1	–	–

^aUnits for rate constants are h^{–1} and the % yield is the average final % yield.^bFold yield increase compared to yield in aqueous buffer.

Table IIYields and Observed Rate Constants for 10DM24^a

Cosolvent Conditions	% Yield	k_{obs}
Aqueous buffer	81	0.34
10% methanol	80	0.20
10% isoamyl alcohol	80	0.27
10% acetone	79	0.27
10% DMSO	81	0.21
10% DMF	77	0.21
Ethanol concentration		
5% ethanol	83	0.3
10% ethanol	81	0.15
20% ethanol	84	0.24
30% ethanol	85	0.24
40% ethanol	83	0.129
50% ethanol	66	0.110
55% ethanol	55	0.10
60% ethanol	34	0.09

^a Units for rate constants are min^{-1} and the % yield is the average final % yield.

Table IIIYields and Observed Rate Constants for 10DM24 and 9DB1 With Decreasing MgCl_2 ^a

MgCl_2 Concentration (mM)	Cosolvent Conditions	10DM24		9DB1	
		% Yield	k_{obs}	% Yield	k_{obs}
40	Aqueous buffer	81	0.34	71	0.121
	20% ethanol	84	0.24	96	1.23
20	Aqueous buffer	79	0.13	28	0.025
	20% ethanol	82	0.17	95	0.72
10	Aqueous buffer	80	0.09	<2	–
	20% ethanol	82	0.12	93	0.265
5	Aqueous buffer	80	0.047	<1	–
	20% ethanol	82	0.055	66	0.084
1	Aqueous buffer	23	0.003	<1	–
	20% ethanol	65	0.012	<1	–

^aUnits for rate constants are min^{-1} for 10DM24 and h^{-1} for 9DB1 and the % yield is the average final % yield.

Table IVYields and Observed Rate Constants for 9DB1^a

Cosolvent Conditions	% Yield	Fold Increase in Yield ^b	k_{obs}
Aqueous buffer	71	–	0.121
10% methanol	93	1.3	0.28
10% isoamyl alcohol	86	1.2	0.122
10% acetone	88	1.2	0.201
10% DMSO	94	1.3	0.229
10% DMF	86	1.2	0.120
Ethanol concentration			
5% ethanol	93	1.3	0.287
10% ethanol	94	1.3	0.36
15% ethanol	95	1.3	0.78
20% ethanol	96	1.3	1.23
30% ethanol	97	1.4	1.80
40% ethanol	82	1.2	0.7
50% ethanol	40	–	0.33
60% ethanol	<5	–	–

^aUnits for rate constants are h^{−1} and the % yield is the average final % yield.^bFold yield increase as compared to yield in aqueous buffer.