Catalytic DNA

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Engineering a Selective Small-Molecule Substrate Binding Site into a Deoxyribozyme**

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Binding of small molecules by nucleic acids contributes to numerous biological processes. For example, riboswitches bind a variety of metabolites to control gene expression,[1] and ribozymes such as the group I intron use an obligatory guanosine cofactor as the nucleophile in the first step of splicing.[2] In several cases, artificial nucleic acid aptamers[3] and enzymes[4] (collectively, functional nucleic acids (FNAs)[5]) have been identified that interact with small molecules.[6,7] In general, understanding the interactions between nucleic acids and small molecules is important for identifying useful FNAs and for rationally extending the scope of their functions.

We recently reported the 7S11 deoxyribozyme, which creates 2′,5′-branched RNA by mediating the attack of an RNA 2′-hydroxy-group nucleophile at a 5′-triphosphate electrophile.[8,9] Both 7S11 and a more general variant, 10DM24,[10] have a three-helix-junction architecture in which the two RNA oligonucleotide substrates and the deoxyribozyme interact through extensive Watson–Crick base pairing (Figure 1a). The 5′-triphosphorylated guanosine electrophile is presented to the branch-site-adenosine nucleophile, which is held at the terminus of the P4 (paired region P4) RNA:DNA helix by Watson–Crick hydrogen bonds. The ligation product is a 2′,5′-branched RNA. Because many natural ribozymes have multi-helix-junction architectures,[11] we are interested to determine the generality of helix-junction platforms for rational deoxyribozyme development. Towards this goal, herein we successfully engineered the 10DM24 deoxyribozyme to mediate the multiple-turnover ligation reaction of a small-molecule nucleoside triphosphate (NTP) rather than a 5′-triphosphorylated oligonucleotide as an electrophilic substrate. We also examined in detail the requirements for productive substrate binding. Hydrogen bonding contributes substantially to selective binding of the NTP, and structural preorganization within the substrate is important for its efficient utilization.

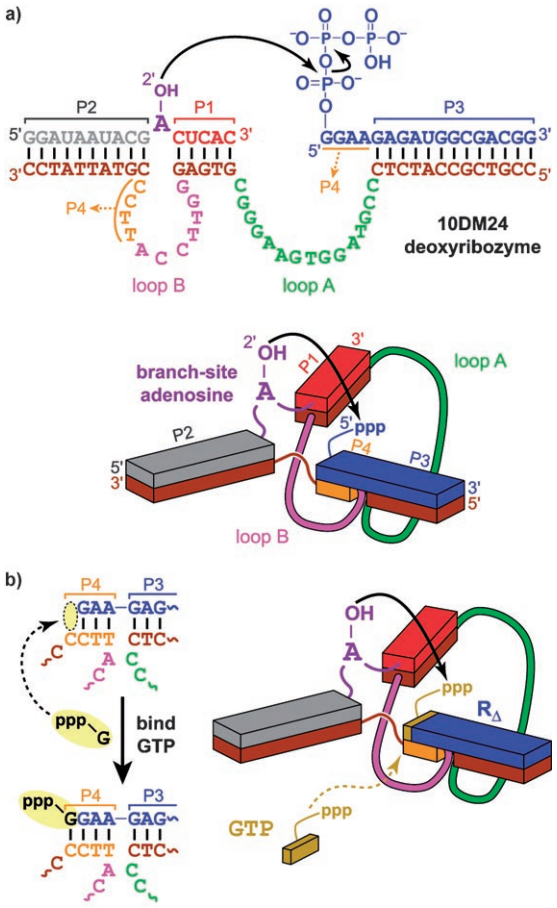

Figure 1. The 10DM24 deoxyribozyme and the use of a small-molecule substrate. a) Secondary structure and schematic three-helix-junction tertiary structure of 10DM24. The detailed three-dimensional structure of the deoxyribozyme-substrate complex is not known. b) Engineering into 10DM24 of a well-defined binding site for GTP as a small-molecule substrate.

We began our experiments with the original 10DM24 arrangement shown in Figure 1a. Conceptually breaking the right-hand (R) oligonucleotide substrate shown in blue immediately to the 3′ side of the first nucleotide leads, in principle, to a deoxyribozyme–substrate complex in which guanosine 5′-triphosphate (GTP) can bind as a discrete electrophile in the location corresponding to the 5′-terminal position of the P4 helix (Figure 1b). We experimentally tested the ability of 10DM24 to catalyze ligation according to this design by using free GTP as a substrate, thereby transferring guanosine 5′-monophosphate (GMP) to the branch-site-adenosine 2′-hydroxy group. When R_Δ, an oligoribonucleotide cofactor that corresponds to all of the remaining nucleotides

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of R, was added to 10DM24 along with GTP, ligation was efficient (Figure 2; 94% yield in 5 h and $k_{obs} = 0.034\ min^{-1}$ under the standard incubation conditions of 1 mM GTP and 40 mM $MgCl_2$ at pH 9.0 and 37 °C). When R_Δ was omitted, no reaction was observed.[12,13] The identity of the PAGE-purified product was confirmed by partial alkaline hydrolysis and MALDI mass spectrometry.[13] From a plot of the k_{obs} value versus the GTP concentration, the $K_{d,app}$ value (apparent dissociation constant) for GTP was found to be greater than 1 mM. The k_{obs} value increased eightfold to $0.26\ min^{-1}$ under the enhanced incubation conditions of 10 mM GTP and 150 mM $MgCl_2$ at pH 9.0 and 37 °C (94% yield in 3 h).

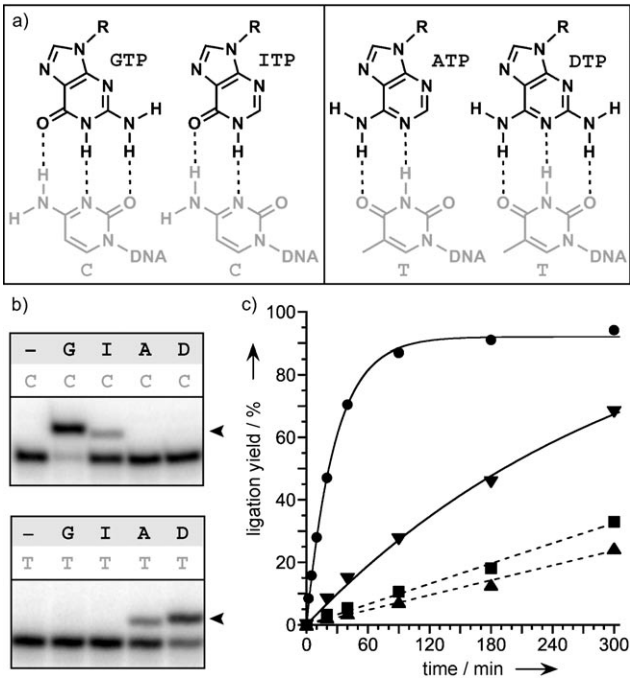

Figure 2. Reaction of a small-molecule NTP substrate catalyzed by the 10DM24 deoxyribozyme. Successful ligation is observed only when the NTP substrate has Watson–Crick complementarity to the terminal P4 DNA nucleotide of 10DM24. a) Watson–Crick interactions between the NTP substrate (top) and the terminal P4 DNA nucleotide of 10DM24 (bottom). b) PAGE images showing the 5-h time point after the ligation reactions were performed with 1 mM of the indicated NTP (– indicates no NTP), 40 mM $MgCl_2$, pH 9.0, and 37 °C. The ligation products are marked with an arrowhead. c) Kinetic plots for Watson–Crick combinations. The points are (top to bottom) GTP ●, DTP ▼, ATP ■, and ITP ▲. The solid lines denote reactions of NTPs that form three Watson–Crick hydrogen bonds with the deoxyribozyme, whereas the dashed lines denote reactions of NTPs that form only two Watson–Crick hydrogen bonds.

We then examined the generality of the ligation reaction by using other NTP substrates in place of GTP. The analogous reaction with the full-length R oligonucleotide as the substrate proceeds well when a 5′-terminal G is present and with only a fivefold lower k_{obs} value than with 5′-A, whereas 5′-C supports greatly reduced activity and 5′-U leads to almost no product (in all cases, the corresponding deoxyribozyme nucleotide is changed to maintain Watson–Crick complementarity).[14] We therefore focused on the purine NTPs (GTP and ATP) and their derivatives. When 1 mM ATP was provided as a small-molecule substrate in place of GTP by using the original 10DM24 sequence and R_Δ, no reaction was observed (< 1% in 5 h). However, when the corresponding deoxyribozyme nucleotide was changed from C to T, substantial ligation was observed with ATP (33% yield in 5 h and $k_{obs} = 0.0008\ min^{-1}$ under standard conditions; Figure 2) but no longer with GTP (< 1% in 5 h). Furthermore, the k_{obs} value increased 16-fold to $0.013\ min^{-1}$ under the enhanced conditions with 10 mM ATP (82% yield in 3 h).[13] These data are as expected for a Watson–Crick base pair between the deoxyribozyme and the NTP substrate.

We varied the number of hydrogen bonds between the NTP substrate and the deoxyribozyme, anticipating that this could influence the efficiency of the ligation reaction. Indeed, the ligation yield and rate increased when 2,6-diaminopurine ribonucleoside triphosphate (DTP) rather than ATP was paired with T in the DNA (Figure 2; 68% in 5 h and $k_{obs} = 0.0032\ min^{-1}$ under standard conditions; 90% in 3 h and $k_{obs} = 0.027\ min^{-1}$ under enhanced conditions).[13] In contrast, when the original C in the deoxyribozyme was retained and inosine triphosphate (ITP) rather than GTP was provided as the substrate, a decrease in activity was observed (25% in 5 h and $k_{obs} = 0.0007\ min^{-1}$ under standard conditions; 84% in 3 h and $k_{obs} = 0.014\ min^{-1}$ under enhanced conditions).[13] Therefore, three hydrogen bonds (GTP, DTP) rather than two hydrogen bonds (ITP, ATP) lead to better activity (solid versus dashed lines in Figure 2c). Perhaps surprisingly, replacing the adenine nucleobase of the substrate with 2-aminopurine led to a tenfold decrease in the k_{obs} value,[13] even though both purine derivatives can form two hydrogen bonds with T in the deoxyribozyme.[15] Therefore, in terms of contribution to NTP substrate binding, the hydrogen bond facing the major groove is more important than the hydrogen bond facing the minor groove.

In addition to the Watson–Crick base pair involving the NTP substrate, the structural model shown in Figure 1b also depicts a base pair at the second position of the P4 helix. Our previous data strongly suggested the presence of this second base pair when a full-length R substrate is used (as in Figure 1a). This was confirmed here with more comprehensive data.[13] We then established that this second base pair is required when using GTP as the substrate with the R_Δ cofactor; furthermore, alteration of the base-pair identity is tolerated with only moderate changes in activity.[13,16] These data, along with the Watson–Crick covariation involving the NTP substrate itself, provide compelling support for the binding model depicted in Figure 1b.[17] To place this Watson–Crick binding mode in context, the other artificial aptamers and nucleic acid enzymes that interact with NTP substrates generally do so through non-Watson–Crick interactions (where the interaction mode is known) with μM to mM binding constants.[6,7] In contrast, the natural purine-binding riboswitches bind their cognate nucleobase through Watson–Crick interactions.[18] In the latter cases, the nucleobase ligands are completely engulfed by the RNA, which enables quite low (nM) dissociation constants. A Watson–Crick binding mode is also observed for the $preQ_1$ riboswitch, which has nM affinity for its ligand.[19] It should be noted that not all

biologically relevant interactions between RNA and substrates are high affinity; for example, the glmS riboswitch binds glucosamine 6-phosphate (GlcN6P) with a $K_{d,app}$ value of merely 0.2 mM.[20]

We evaluated changes to the small-molecule NTP substrates that probe the role of the ribose ring, including potential effects of structural preorganization.[13] Both 2′-deoxyGTP (dGTP) and 2′,3′-dideoxyGTP (ddGTP) are tolerated well, with no diminution of yield and at most a threefold decrease in the k_{obs} value relative to GTP. Similarly, arabino-ATP (which has the opposite 2′-configuration relative to ATP), dATP, and ddATP all have a k_{obs} value within twofold of ATP itself. From these data, we conclude that the deoxyribozyme does not require the 2′- or 3′-hydroxy groups, nor does it directly contact either the 2′- or 3′-hydrogens of the ribose ring. We additionally considered how perturbations in the structural preorganization of the substrate impact the ligation activity by using two substrate analogues (Figure 3a). First, in place of GTP we used C2–C3-cleaved GTP ($G^{clv}TP$), which lacks the C2–C3 bond of the ribose ring but has the same number of heavy (non-hydrogen) atoms.[21] Second, in place of GTP we used acyclovir triphosphate ($G^{acv}TP$), where acyclovir is the guanosine analogue that lacks both the C2 and C3 carbons and hydroxy groups of the ribose ring.[22] For both $G^{clv}TP$ and $G^{acv}TP$, only a very small amount of ligation activity was observed (Figure 3b); the k_{obs} value was diminished relative to GTP by approximately 1000-fold ($G^{clv}TP$) or 300-fold ($G^{acv}TP$).[13] The products were isolated by PAGE; all had the expected connectivity, as confirmed by partial alkaline hydrolysis (Figure 3c). By design, the nucleobase and triphosphate (i.e., recognition and reactive) moieties of $G^{clv}TP$ and $G^{acv}TP$ are not structurally constrained by the five-membered ribose ring that is present within GTP itself. Therefore, the poor reactivities of these two modified substrates demonstrate that the preorganization enforced by the ribose ring of GTP contributes substantially to the efficiency of the deoxyribozyme-catalyzed ligation reaction.

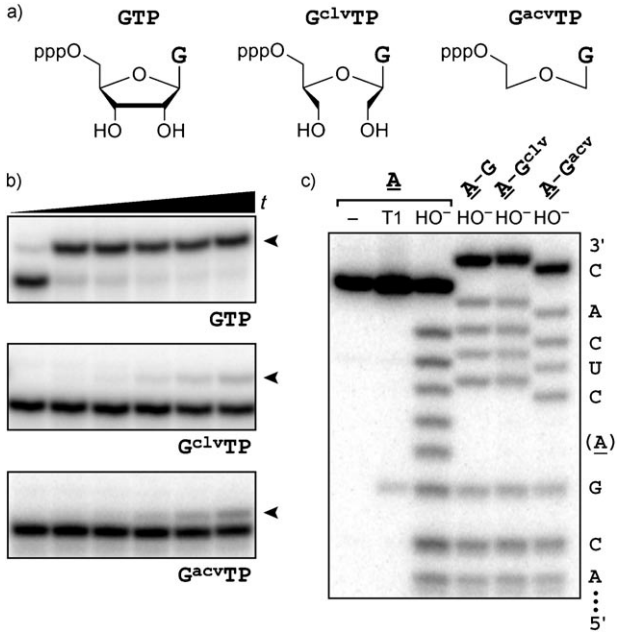

Figure 3. Evaluation of the role of structural preorganization within the substrate. a) Structures of GTP, cleaved GTP ($G^{clv}TP$), and acyclovir triphosphate ($G^{acv}TP$). b) Ligation assays with GTP and the two analogues. Data were obtained with 10 mM NTP (or analogue), 150 mM $MgCl_2$, pH 9.0, and 37 °C. The ligation products are marked with an arrowhead. c) Partial alkaline hydrolysis demonstrates the expected connectivity for each product. The branch-site adenosine is underlined. The partial alkaline hydrolysis (HO^-) ladder was assigned by comparison with the RNase T1 cleavage ladder (T1).

All of our previous deoxyribozyme-mediated ligation reactions with two RNA oligonucleotide substrates had displayed only single-turnover ligation behavior, which was attributed to product inhibition (similar to natural protein enzymes that ligate nucleic acids).[23] In contrast, upon the 10DM24-catalyzed reaction of the oligoribonucleotide 2′-hydroxy group with the NTP substrate, the binding affinity of the RNA for the deoxyribozyme is not expected to increase substantially. On this basis, we anticipated that multiple turnover should be observable in the engineered deoxyribozyme–NTP system. Indeed, with GTP as the substrate, we were for the first time able to observe unambiguous multiple-turnover behavior by using an RNA ligase deoxyribozyme (five turnovers were observed in 5 h).[13]

Finally, we showed that a binding site for an NTP cofactor can be located adjacent to the substrate binding site. This was achieved by removing an additional nucleotide from the R_Δ cofactor (blue in Figure 1b), forming the shorter $R_{\Delta\Delta}$ cofactor and requiring two added nucleotides to reconstitute the complete P4 region (Figure 4a). When GTP was incubated with the parent 10DM24 deoxyribozyme (which has 3′-CCTT-5′ in its P4 region) along with the $R_{\Delta\Delta}$ cofactor and the oligoribonucleotide that provides the 2′-hydroxy group, a product band matching the branched standard was observed. Additionally, a small amount of a more slowly migrating product was also observed (Figure 4b, top section, lane 3). Partial alkaline hydrolysis of both products revealed that they are branched with respective connectivities A–G and A–GG (where A is the branch-site adenosine).[13] We hypothesized that the new A–GG product is formed by initial templated but otherwise uncatalyzed synthesis of a GG dinucleotide (i.e., pppGpG) from two GTP molecules followed by 10DM24-catalyzed branch formation by using this dinucleotide.[24] Consistent with this hypothesis, the purified A–G product was unreactive with GTP when resubjected to the reaction conditions,[13] which suggests that the two G nucleotides are not attached successively. The pppGpG dinucleotide was synthesized independently by using T7 RNA polymerase[6,13] and, as expected, led solely to the A–GG product (Figure 4b, top section, lane 4; also as expected, the $R_{\Delta\Delta}$ cofactor was required to observe this product[13]). Although the pppGpG substrate had a $K_{d,app}$ value of greater than 1 mM with $R_{\Delta\Delta}$, similar to the $K_{d,app}$ value for GTP with R_Δ,[13] the ligation reaction with pppGpG and $R_{\Delta\Delta}$ had a k_{obs} value that was sixfold higher than for the analogous reaction with GTP and R_Δ (Figure 4c).

When similar experiments were performed by using the mutant 10DM24 deoxyribozyme that has CTTT rather than CCTT in the P4 region, the A–GG product was not observed when using GTP (Figure 4b, bottom section, lane 3). How-

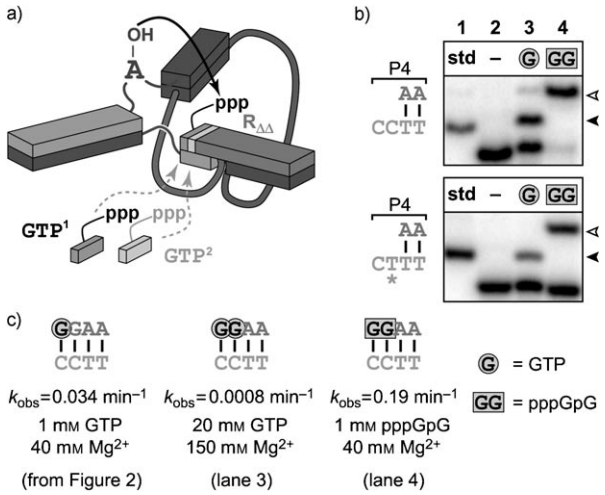

Figure 4. Use of a second NTP as a cofactor for the ligation reaction. a) Schematic depiction; compare with Figure 1b. b) The shortened P4-region sequence is depicted adjacent to each set of gel lanes (the asterisk in the bottom section denotes the single altered DNA nucleotide). The standard (lane 1) is the branched product made by using GTP as the substrate with R_Δ (see Figure 2b, G–C lane). The 5-h time points were obtained at pH 9.0 and 37 °C. In lane 3, 20 mM GTP and 150 mM $MgCl_2$ were also included. In lane 4, 1 mM pppGpG and 40 mM $MgCl_2$ were also included. The branched products are assigned as A–G (filled arrowhead) and A–GG (open arrowhead) and the branch-site adenosine is underlined. c) Comparison of k_{obs} values for GTP or pppGpG as the substrate when aligned in several combinations.

ever, the separately prepared pppGpG dinucleotide still led to the A–GG product (Figure 4b, bottom section, lane 4), but with a ~140-fold lower efficiency.[13] Although a G–C base pair is preferred, G can occupy the second position of the P4 helix regardless of the deoxyribozyme nucleotide across from it.[13] The data with the mutant deoxyribozyme suggest that binding at the second P4 position of GTP in the G–T wobble geometry strongly disfavors templated GG dinucleotide synthesis, but still permits slow reaction of the branch-site adenosine 2′-hydroxy group with the GTP molecule bound as the substrate at the first P4 position.

In summary, we have shown that the three-helix-junction architecture of the 10DM24 deoxyribozyme fosters rational engineering of a selective binding site for a small-molecule NTP substrate that reacts in multiple-turnover fashion. In analogy to the natural purine-binding riboswitches, the selectivity of 10DM24 for its NTP substrate is enforced by Watson–Crick hydrogen bonding. Structural preorganization within the small-molecule substrate is important for catalytic activity of the deoxyribozyme, and a cofactor binding site can be introduced adjacent to the substrate binding site. These results establish a detailed baseline for further rational approaches to identify and improve the functions of nucleic acid enzymes. Utilization of an NTP substrate by the 10DM24 deoxyribozyme may also have practical value. For example, the nucleotide that is attached to the RNA substrate by the 10DM24 deoxyribozyme has a 2′,3′-diol moiety that is susceptible to periodate oxidation and subsequent derivatization, which is analogous to long-established approaches for labeling of the RNA 3′ terminus.[25] Therefore, deoxyribozyme-catalyzed internal attachment to RNA of a small-molecule NTP substrate will likely form the basis of a succinct and nonperturbing site-specific RNA modification strategy.[27] It should also be possible to use covalently modified NTPs directly as ligation substrates. This may require selection in vitro of new deoxyribozymes that accept such substrates.

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[13] See the Supporting Information for all data that is not shown in the figures.
[14] This trend was reported for 7S11 by using 5′-adenylated substrates,[9] and a similar trend is observed with 10DM24 (see the Supporting Information).
[15] For practical reasons, these experiments were performed with the 2′-deoxy-NTPs (i.e., dATP and d2AP-TP, where 2AP is 2-aminopurine). Because dATP is almost as efficient a substrate as ATP (see below), the 2′-deoxy modification of d2AP-TP is not responsible for its poor reactivity as a substrate relative to dATP.
[16] The identity of the second base pair could in principle influence the NTP binding affinity through stacking or other effects.
[17] A modest amount of ligation activity was observed when GTP or ITP was used along with A in the deoxyribozyme (see the Supporting Information). This indicates that non-Watson–Crick interactions between the NTP substrate and deoxyribozyme are possible, albeit much less productive than Watson–Crick interactions.
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