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Pursuing DNA Catalysts for Protein Modification

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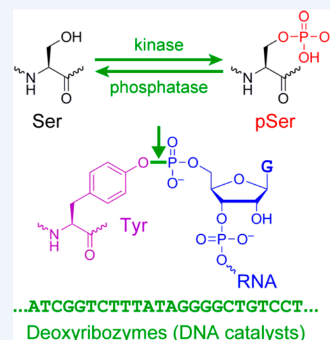
CONSPECTUS: Catalysis is a fundamental chemical concept, and many kinds of catalysts have considerable practical value. Developing entirely new catalysts is an exciting challenge. Rational design and screening have provided many new small-molecule catalysts, and directed evolution has been used to optimize or redefine the function of many protein enzymes. However, these approaches have inherent limitations that prompt the pursuit of different kinds of catalysts using other experimental methods.



Nature evolved RNA enzymes, or ribozymes, for key catalytic roles that in modern biology are limited to phosphodiester cleavage/ligation and amide bond formation. Artificial DNA enzymes, or deoxyribozymes, have great promise for a broad range of catalytic activities. They can be identified from unbiased (random) sequence populations as long as the appropriate in vitro selection strategies can be implemented for their identification. Notably, in vitro selection is different in key conceptual and practical ways from rational design, screening, and directed evolution. This Account describes the development by in vitro selection of DNA catalysts for many different kinds of covalent modification reactions of peptide and protein substrates, inspired in part by our earlier work with DNA-catalyzed RNA ligation reactions.

In one set of studies, we have sought DNA-catalyzed peptide backbone cleavage, with the long-term goal of artificial DNA-based proteases. We originally anticipated that amide hydrolysis should be readily achieved, but in vitro selection instead surprisingly led to deoxyribozymes for DNA phosphodiester hydrolysis; this was unexpected because uncatalyzed amide bond hydrolysis is 10^5-fold faster. After developing a suitable selection approach that actively avoids DNA hydrolysis, we were able to identify deoxyribozymes for hydrolysis of esters and aromatic amides (anilides). Aliphatic amide cleavage remains an ongoing focus, including via inclusion of chemically modified DNA nucleotides in the catalyst, which we have recently found to enable this cleavage reaction. In numerous other efforts, we have investigated DNA-catalyzed peptide side chain modification reactions. Key successes include nucleopeptide formation (attachment of oligonucleotides to peptide side chains) and phosphatase and kinase activities (removal and attachment of phosphoryl groups to side chains).

Through all of these efforts, we have learned the importance of careful selection design, including the frequent need to develop specific "capture" reactions that enable the selection process to provide only those DNA sequences that have the desired catalytic functions. We have established strategies for identifying deoxyribozymes that accept discrete peptide and protein substrates, and we have obtained data to inform the key choice of random region length at the outset of selection experiments. Finally, we have demonstrated the viability of modular deoxyribozymes that include a small-molecule-binding aptamer domain, although the value of such modularity is found to be minimal, with implications for many selection endeavors.

Advances such as those summarized in this Account reveal that DNA has considerable catalytic abilities for biochemically relevant reactions, specifically including covalent protein modifications. Moreover, DNA has substantially different, and in many ways better, characteristics than do small molecules or proteins for a catalyst that is obtained "from scratch" without demanding any existing information on catalyst structure or mechanism. Therefore, prospects are very strong for continued development and eventual practical applications of deoxyribozymes for peptide and protein modification.

■ INTRODUCTION

Chemists generally follow one of two strategies when developing new catalysts. The "small-molecule" approach uses a combination of rational design and screening to identify low-molecular-weight catalysts that are collectively applicable for a very broad range of reactivity and under a plethora of reaction conditions.[1,2] Small-molecule catalysts feature elements from the entire periodic table and have almost no constraints on their chemical compositions. Alternatively, the "directed evolution" approach begins with naturally occurring protein enzymes and evolves their amino acid sequences for improved properties such as rate constant and selectivity or in some cases for catalysis of different but mechanistically related chemical reactions.[3,4]

Certain chemical transformations are not readily accomplished using either small-molecule catalysts or evolved protein enzymes. Examples of such reactions include various side chain modifications of unprotected protein substrates, especially when seeking site selectivity among the common side chains. Small-molecule catalysts cannot always achieve high selectivity, in part because their size does not allow them to discriminate

Received: February 17, 2015
Published: May 5, 2015

ACS Publications © 2015 American Chemical Society

DOI: 10.1021/acs.accounts.5b00090
Acc. Chem. Res. 2015, 48, 1369–1379

the sequence contexts of, e.g., two serine side chain hydroxyl groups when presented on a protein surface. Also, small-molecule catalysts may not tolerate the aqueous conditions required to function with most protein substrates. Evolved protein enzymes have achieved many successes, but if nature has not provided a reasonably good enzymatic starting point for a desired catalytic activity, then directed evolution cannot be implemented.

Our laboratory uses principles somewhat related to, but distinct from, those of directed evolution to identify particular DNA sequences that have catalytic activity. Natural RNA enzymes, or "ribozymes", were discovered in the early 1980s and have been studied intensively for their ability to catalyze RNA phosphodiester cleavage/ligation reactions as well as peptide bond formation in the ribosome.[5,6] By analogy, artificial DNA enzymes are called "deoxyribozymes" or "DNAzymes". Although DNA exists primarily in double-stranded form in biology, single-stranded DNA can adopt secondary and tertiary structure (just like single-stranded RNA) and therefore can be catalytic. Deoxyribozymes enjoy many practical advantages related to stability, cost, and ease of synthesis compared with both RNA and proteins. DNA catalysts are not known to exist in nature; any natural DNA enzymes are likely rather limited in reaction scope. The scope of unnatural DNA catalyst function is an experimental question that is a main focus of our research.

When developing enzyme-like catalysts, one can consider all of the above approaches, i.e., rational design, screening, and directed evolution. Rational design of *protein* enzyme active sites is conceptually possible although practically challenging. Several successes have relied upon directed evolution for experimental optimization or even complete remodeling of computed active-site structures, which are embedded in robust surrounding structures of unrelated proteins.[7−9] At present, such approaches are untenable for *nucleic acid* catalysts, and their prospects are uncertain in part because of poor current understanding of active-site structure and function. One-at-a-time screening approaches are inherently unlikely to succeed for either protein or nucleic acid enzymes given the immense size of sequence space, which increases as 20^n for proteins and 4^n for nucleic acids, where n is the length of the sequence. Some quantitative considerations of sequence space have been discussed in a previous review.[10] Finally, because of the lack of natural starting points, we cannot in general implement "directed evolution of DNA enzymes" unless we begin with the sequence of one of the rather small number of known artificial deoxyribozymes. This problem is particularly acute when seeking entirely new DNA catalyst function, considering the rather limited range of function (i.e., reaction types) reported to date.

Fortunately, in vitro selection methods[11,12] are capable of identifying ribozymes and deoxyribozymes starting from entirely unbiased (random) DNA sequence populations, or "pools", with no preconceived notion of catalyst structure or mechanism. In contrast, in vitro selection is difficult to implement with proteins for both conceptual and methodological reasons.[13] Although in vitro selection can certainly be performed with ribozymes,[14] DNA has many practical advantages over RNA without any clear functional disadvantage because of the "missing" 2′-hydroxyl group.[15] The first deoxyribozyme was reported in 1994 for cleavage of an RNA linkage,[16] and since then, a growing variety of DNA-catalyzed reactions have been described. This Account is not a comprehensive review of deoxyribozymes[17] or in vitro selection methodology but is instead an update on Silverman laboratory research on the development of DNA catalysts for new reactions. The focus is on our progress since our previous Account in 2009, which described primarily our studies of DNA-catalyzed RNA ligation.[18] Since then, we have broadened our efforts especially to seek DNA-catalyzed covalent modifications of peptide and protein substrates, with an eye toward practical applications of the corresponding deoxyribozymes.

FROM RNA CLEAVAGE TO RNA LIGATION TO CURRENT EFFORTS

Our earliest studies with deoxyribozymes focused on RNA ligation, i.e., the joining of two RNA substrates. RNA ligation is superficially the "opposite" of the earlier-achieved RNA cleavage by transesterification, i.e., mediating attack of the RNA 2′-hydroxyl group at the adjacent phosphodiester (Figure 1). However, RNA ligation is more challenging to achieve because the two reactive functional groups (of which several combinations may be chosen) are not highly preorganized or at least proximal, as is inherently the case for RNA cleavage. Our initial motivation in pursuing RNA ligation was to provide an alternative means of preparing large site-specifically modified RNAs for studies of RNA structure and folding. The previous Account summarizes much of these efforts on DNA-catalyzed synthesis of linear and branched RNA,[18] and we published a few RNA ligation articles after that time. Others have subsequently used RNA ligase deoxyribozymes as enabling tools in biochemical and biophysical studies of RNA.[19−22] Because prospects were strong for developing DNA catalysts for many reactions beyond RNA ligation, we endeavored to include substrates other than RNA.

DETOUR TO DNA-CATALYZED DNA HYDROLYSIS

Noting the great success of DNA-catalyzed RNA cleavage, we considered that DNA-catalyzed amide bond hydrolysis should be possible, and artificial DNA catalysts with protease activity would have numerous practical applications. The uncatalyzed half-lives for cleavage of individual RNA and protein linkages are only about 20-fold different, with $t_{1/2}$ values of ∼10 years for RNA cleavage by transesterification[23] and 200 years for peptide bond hydrolysis (Figure 2).[24] Because DNA can catalyze RNA cleavage on the time scale of minutes, we anticipated that DNA should also be able to catalyze amide bond hydrolysis on the time scale of hours if not minutes. We therefore prepared a substrate with a tripeptide segment located

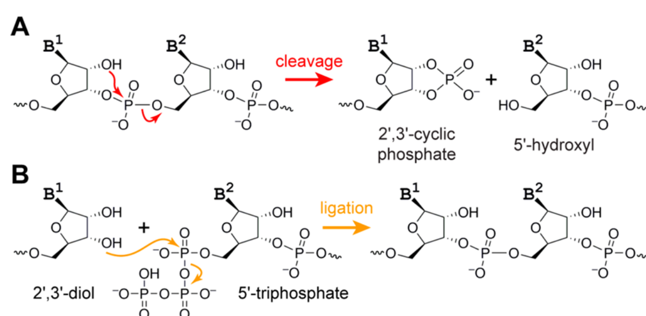


Figure 1. RNA cleavage and ligation reactions. (A) RNA cleavage by transesterification. (B) RNA ligation, illustrated with 3′-hydroxyl + 5′-triphosphate. The reverse of the RNA cleavage reaction in (A) is also possible.

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Acc. Chem. Res. 2015, 48, 1369−1379

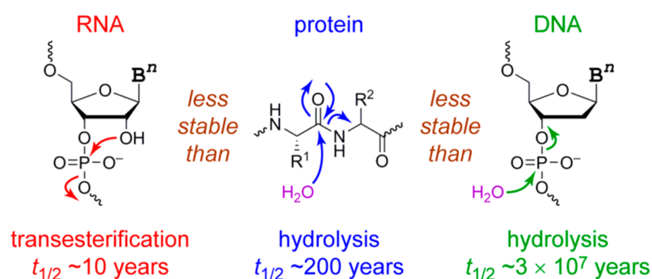

Figure 2. Uncatalyzed half-lives of individual linkages in RNA,[23] protein,[24] and DNA[26] (at near-neutral pH and near-ambient temperature in the absence of divalent metal ions).

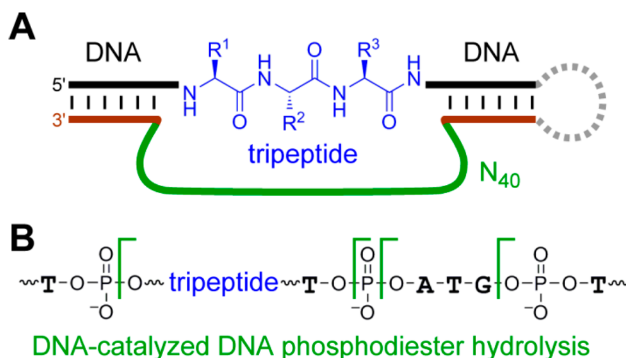

Figure 3. Initial foray toward DNA-catalyzed peptide bond cleavage and unexpected identification of DNA catalysts for DNA phosphodiester hydrolysis.[25] (A) Selection design with a tripeptide substrate embedded between two DNA binding arms. (B) Selection outcome of DNA-catalyzed DNA phosphodiester hydrolysis. Each deoxyribozyme catalyzes hydrolysis specifically at one of the marked phosphodiester bonds.

between two DNA binding arms (Figure 3A), analogous to the earliest studies of DNA-catalyzed RNA cleavage that used a substrate with a ribonucleotide linkage between two DNA binding arms. The basis for in vitro selection was a downward polyacrylamide gel electrophoresis (PAGE) shift specifically for those DNA sequences in the N_{40} (40 nucleotides long) random pool that catalyze cleavage of their attached substrate. Upon selection intended to provide cleavage of the peptide, we were surprised to discover deoxyribozymes that instead catalyze sequence-specific hydrolysis of DNA phosphodiester linkages (Figure 3B).[25] The uncatalyzed half-life of DNA is ~30 million years,[26] ~10^5-fold greater than that for protein, highlighting the unexpected nature of this finding. The possibility of oxidative cleavage rather than hydrolysis of DNA was addressed by a key experiment with $H_2{}^{18}O$. The ^{18}O became incorporated into the DNA product, thereby providing firm evidence for hydrolysis.

Subsequent to our initial report, we explored many aspects of DNA-catalyzed DNA hydrolysis. These efforts are not described here, except to summarize that we explored the substrate sequence dependence, site selectivity, and metal ion dependence.[27−29] In these efforts, we were consistently able to identify DNA catalysts that hydrolyze single-stranded DNA substrates, where the deoxyribozyme binds to the substrate via easily programmed Watson−Crick binding interactions. However, the truly valuable practical objective would be DNA-catalyzed hydrolysis of *double*-stranded DNA, which would require either (1) strand invasion by deoxyribozymes to separate the two substrate strands followed by independent cleavage of each single strand or (2) direct binding and cleavage of double-stranded DNA without the benefit of Watson−Crick binding interactions by the deoxyribozyme. In numerous unpublished efforts we pursued both of these strategies, unfortunately without success. In the meantime, the increasingly apparent practical value of DNA cleavage tools such as ZFNs, TALENs, and most recently CRISPR-Cas9[30,31] led us away from DNA-catalyzed DNA hydrolysis and toward catalysis by DNA with protein substrates.

Along the way, we used DNA-catalyzed phosphodiester hydrolysis to explore an interesting question: can DNA catalyze an otherwise-disfavored chemical reaction? In particular, we asked whether DNA can catalyze cleavage of an RNA substrate by hydrolysis rather than transesterification even when the latter pathway is possible (Figure 4).[32] RNA cleavage by group I and group II introns is analogous to RNA hydrolysis rather than transesterification, but these naturally evolved ribozymes are an order of magnitude larger than our typical artificial deoxyribozymes. Our results clearly established that indeed DNA can direct a reaction down a disfavored pathway (RNA hydrolysis) even when a more chemically favorable mechanism (transesterification) is available. This important result provided direct evidence that DNA can induce a reaction that would otherwise not have happened at all in the absence of a catalyst, which should embolden future efforts to seek DNA catalysis of "difficult" reactions.

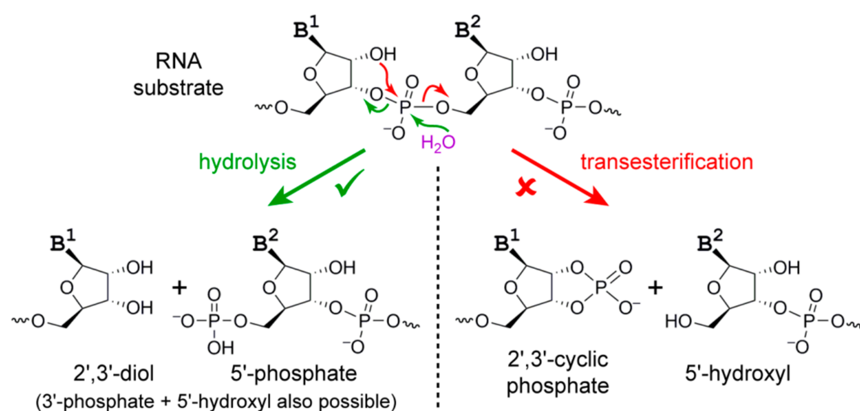

Figure 4. DNA can catalyze an otherwise-disfavored reaction, RNA cleavage by hydrolysis rather than transesterification.[32] Without catalysis, transesterification is favored by >10^6-fold.

DOI: 10.1021/acs.accounts.5b00090
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PURSUING DNA-CATALYZED CLEAVAGE OF AMIDE (PEPTIDE) BONDS

From the efforts described above, an important lesson is applicable to nearly all of our subsequent studies with many DNA-catalyzed reactions. This lesson is the need to include explicit selection pressure that differentiates between desired and undesired reaction outcomes. For this purpose, we developed the concept of a "capture" reaction, i.e., a reaction used during each selection round that specifically separates, or captures, those DNA sequences that have just catalyzed the particular reaction that we seek. In all cases, the key challenge lies in devising and implementing an in vitro selection strategy (including a capture reaction when necessary) that enables the emergence of only those DNA sequences that have the desired catalytic activity, while simultaneously suppressing the survival of sequences that lack this activity.

One product of amide bond hydrolysis is a carboxylic acid, which is not formed by DNA phosphodiester hydrolysis. Therefore, we optimized a capture reaction that joins a 5′-amino "capture oligonucleotide" selectively to a carboxylic acid (Figure 5A). In vitro selection using this capture reaction in each selection round led to several deoxyribozymes for hydrolysis of esters and others for hydrolysis of aromatic amides (anilides) but none for hydrolysis of aliphatic amides (Figure 5B).[33] Because several anilide-hydrolyzing deoxyribozymes were found to have observed rate constant (k_{obs}) values that depend very little on substituents placed on the aromatic ring, we concluded that nucleophilic attack into the aliphatic amide carbonyl group is rate-determining for cleavage. The absence of deoxyribozymes for aliphatic amide hydrolysis means that water must be an insufficiently strong nucleophile in the DNA-catalyzed context, even when in the form of a metal-bound hydroxide anion. Therefore, our ongoing efforts are seeking to use more reactive nucleophiles (such as various nitrogen derivatives) in DNA-catalyzed peptide cleavage reactions analogous to hydrolysis.

In parallel and spurred by others' use of chemically modified nucleotides to aid DNA-catalyzed RNA cleavage (e.g., refs 34 and 35 among others), we have endeavored to identify peptide-hydrolyzing deoxyribozymes that incorporate functional groups other than those normally found in nucleic acids, especially those found in protein enzymes.[36] In particular, we are evaluating the potential contributions of amino-, hydroxyl-, carboxyl-, and imidazolyl-modified DNA nucleotides to amide bond hydrolysis (Figure 6). One can envision a plausible mechanism by which each such functional group could assist catalysis. In preliminary studies, we have obtained unpublished evidence that including modified DNA nucleotides during selection enables aliphatic amide hydrolysis. We are excited to follow up on these findings, which suggest that the synthetic challenges of including modified nucleotides may be rewarded by the resulting catalytic activity.

DNA-CATALYZED SYNTHESIS OF NUCLEOPEPTIDES

The first successful DNA-catalyzed reaction with a peptide-containing substrate was the formation of a nucleopeptide linkage, i.e., a covalent bond between a peptide side chain and an oligonucleotide (Figure 7A).[37] In part, we targeted this reaction as a sensible transitional goal between all-nucleic acid substrates (as in our prior studies with RNA ligation) and peptide/protein substrates. We also noted that nucleopeptides

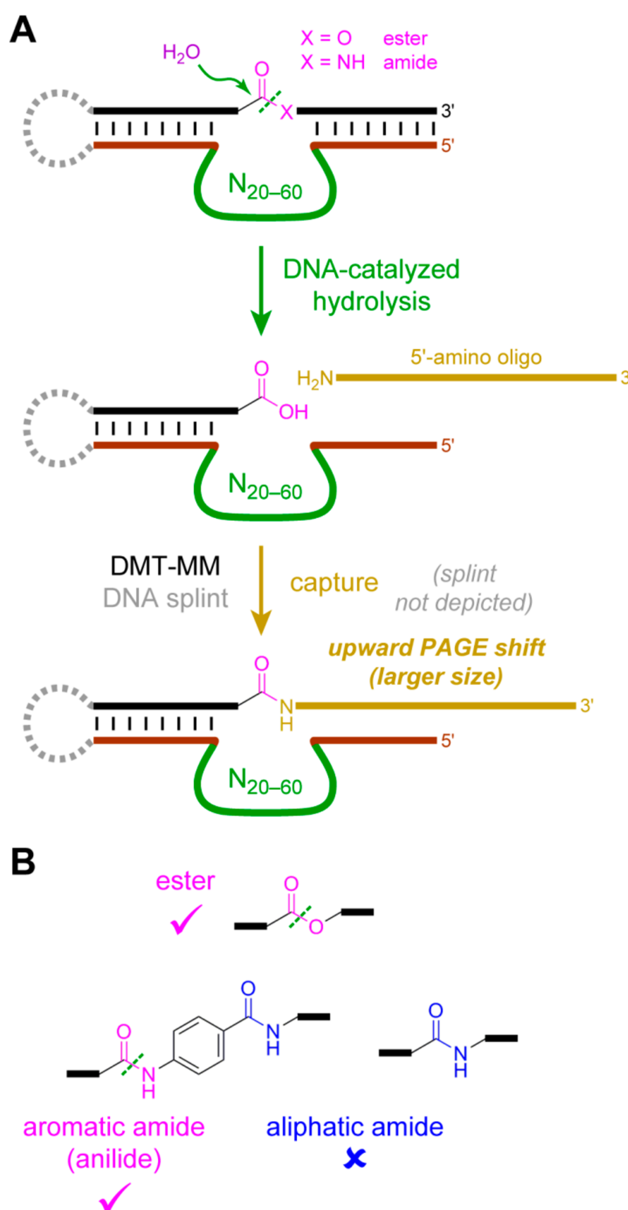

Figure 5. DNA-catalyzed ester and amide cleavage.[33] (A) Capture reaction for the carboxylic acid product of ester or amide hydrolysis. (B) Outcome of selection. We observed DNA-catalyzed hydrolysis of aromatic amide (anilide) substrates but not of aliphatic amides.

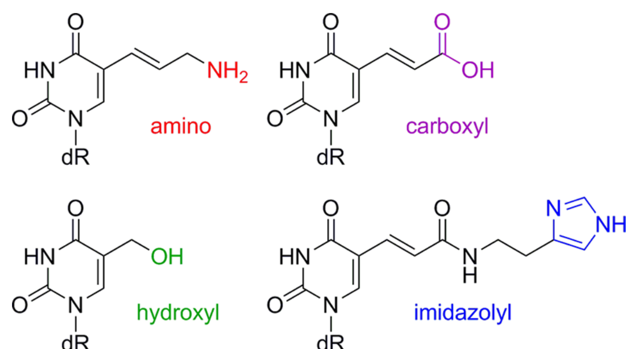

Figure 6. Structures of chemically modified nucleotides for enhancing DNA catalyst function.

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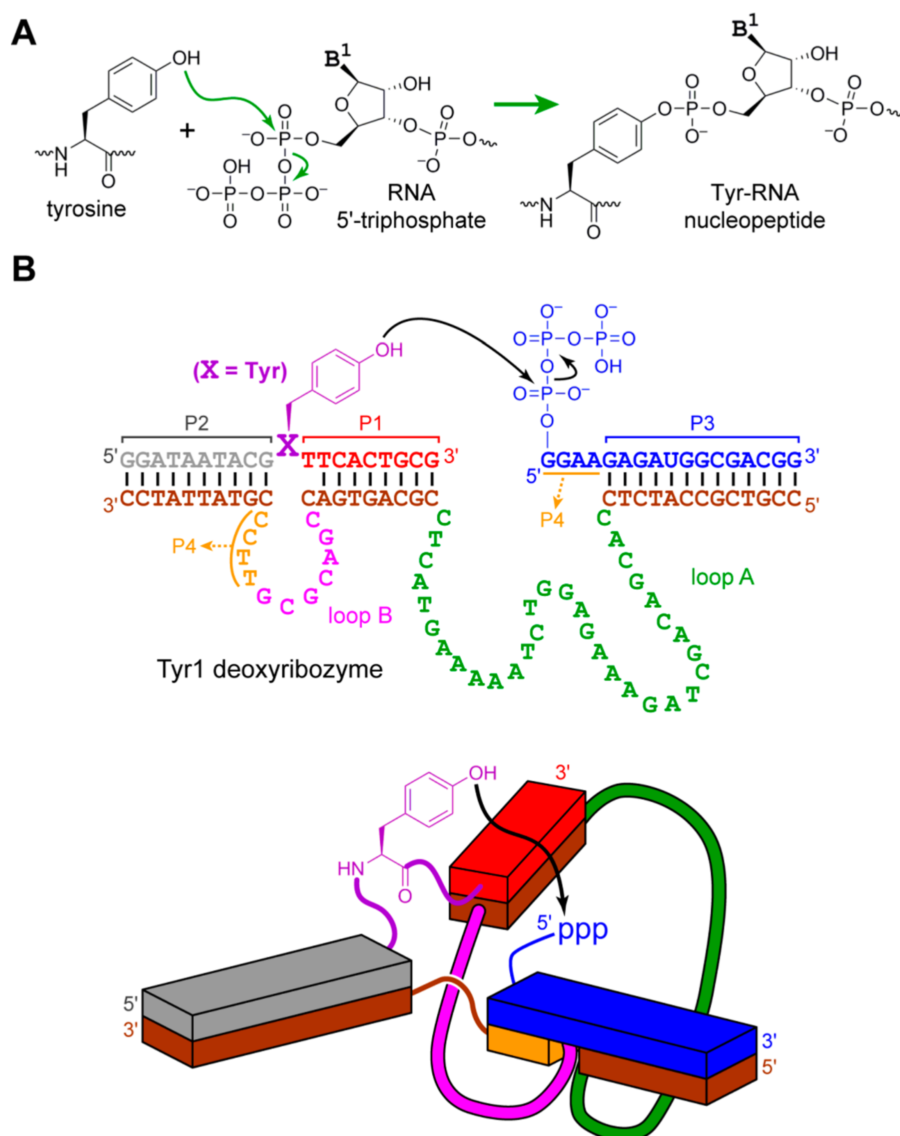

Figure 7. DNA-catalyzed nucleopeptide formation.[37] (A) Reaction between tyrosine and RNA to form a nucleopeptide. (B) Two schematic depictions of the 3HJ architecture that juxtaposes the nucleophile (peptide side chain) and electrophile (RNA 5′-triphosphate).

are formed naturally in several contexts and therefore that their DNA-catalyzed synthesis could be useful. Because the 5′-triphosphorylated RNA electrophile was already validated for DNA catalysis through our studies of RNA ligation, our initial focus was on securing DNA-catalyzed reactivity of nucleophilic peptide side chains.

In previous RNA ligation experiments, we identified deoxyribozymes that were discovered to form a three-helix junction (3HJ) architecture with their two RNA substrates.[38,39] We exploited the 3HJ via preorganization of a peptide side chain nucleophile and the 5′-triphosphate electrophile (Figure 7B). Selection for DNA catalysis in this preorganized 3HJ architecture led first to deoxyribozymes for tyrosine (aromatic −OH group) side chain reactivity when the substrate contained a single tyrosine amino acid[37] and subsequently for serine (aliphatic −OH) reactivity when the substrate included a tripeptide segment.[40] However, lysine ($-NH_2$) reactivity was not observed; another functional group (a phosphoramidate) was observed to react instead of the primary amino group of lysine.[41] These efforts were significant because they demonstrated that DNA can catalyze reactions involving at least hydroxyl-bearing amino acid side chains. However, the findings inherently could not be extended to our longer-term goal of discrete (free) peptides and proteins as substrates because of the requirement for the constrained 3HJ architecture.

AGAIN NUCLEOPEPTIDES: USE OF DISCRETE (FREE) PEPTIDES AS SUBSTRATES

Dispensing with the 3HJ architecture, we began to use peptide substrates attached to a DNA anchor oligonucleotide through a covalent tether (Figure 8). The DNA anchor provides substantial binding energy while still requiring the deoxyribozyme to interact meaningfully with the peptide, especially when the tether is lengthy. In our first such effort, we performed in vitro selection using a tethered tyrosine-containing peptide (Figure 8A) and found that the resulting deoxyribozymes can also catalyze reactions of a discrete (untethered, free) peptide substrate, albeit with millimolar peptide Michaelis constant (K_M).[42]

These results encouraged us to develop a strategy that uses a discrete peptide directly during selection, so that deoxyribo-

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Acc. Chem. Res. 2015, 48, 1369−1379

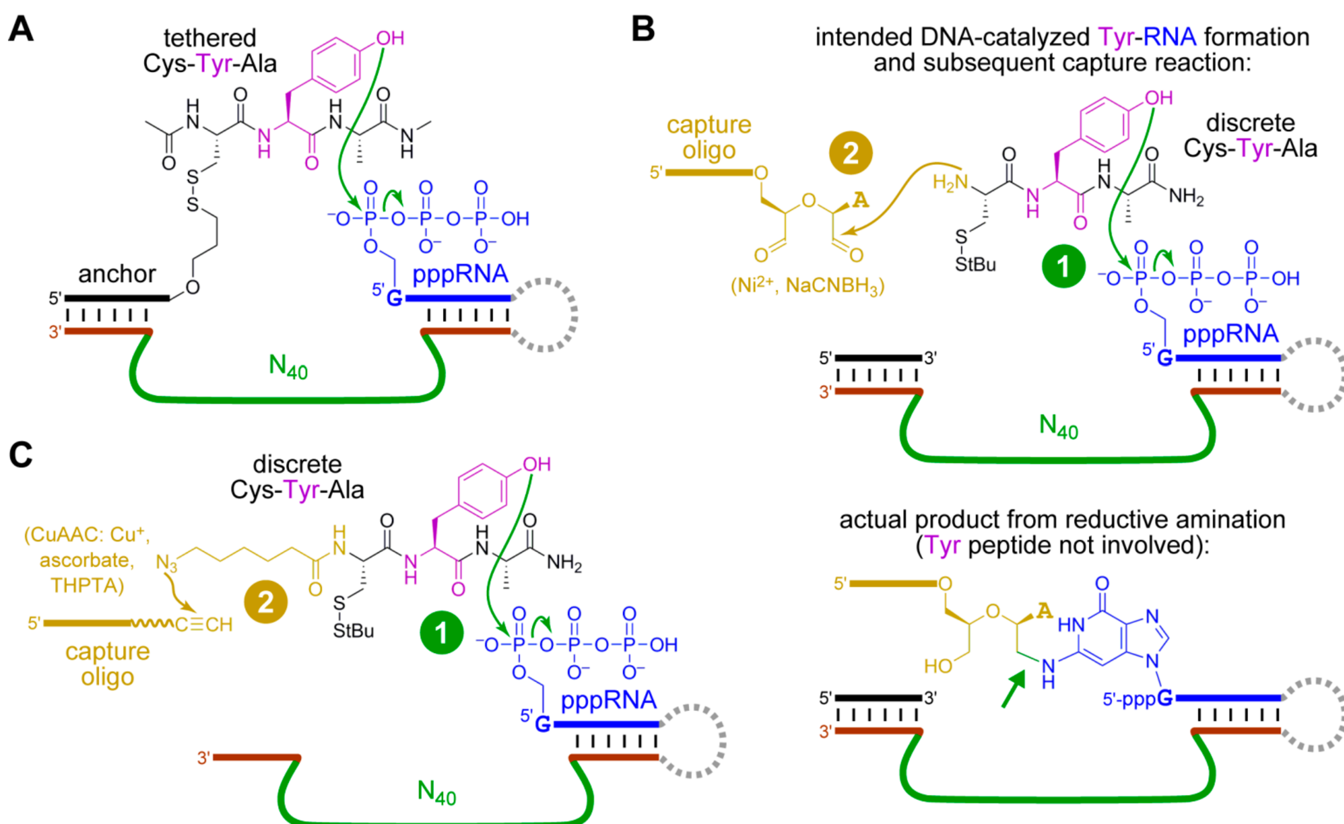

Figure 8. Nucleopeptide formation with a discrete peptide substrate. (A) Tethered peptide substrate and selection arrangement.[42] Various tether compositions and lengths were used; a short tether is illustrated. (B) Selection with a discrete amino peptide and reductive amination capture; the outcome of nucleobase reductive amination.[43] (C) Selection with a discrete azido peptide, CuAAC capture, and the outcome of nucleopeptide formation.[44]

zymes are required to interact more tightly with the unanchored peptide in order to survive the selection process. For this purpose, we initially used a peptide with a free α-amino N-terminus, for which postselection capture by reductive amination (using a 3′-dialdehyde oligonucleotide formed by periodate oxidation of a 3′-ribonucleotide terminus) allows PAGE separation of catalytic DNA sequences. However, the unexpected outcome was deoxyribozymes that catalyze peptide-independent reductive amination of the 5′-terminal guanosine nucleobase with the 3′-dialdehyde in the capture step, which was not intended to be a DNA-catalyzed step (Figure 8B).[43] We therefore instead performed selection using a tyrosine-containing peptide with an azido-modified N-terminus and subsequent copper-catalyzed azide−alkyne cycloaddition (CuAAC) capture with an alkyne-functionalized oligonucleotide, thereby finding deoxyribozymes that function reasonably well with discrete peptides (Figure 8C).[44] This new strategy enabled us to impose selection pressure for catalysis at relatively low peptide concentrations simply by decreasing the peptide concentration during successive selection rounds, which is an impossible approach with a tethered peptide substrate that is covalently attached to each deoxyribozyme candidate. Using the CuAAC strategy, we found one deoxyribozyme with peptide K_M of ~100 μM that was preparatively useful at merely 10 μM peptide, thereby validating the overall approach. Currently we are expanding these efforts to obtain deoxyribozymes for the preparation of both RNA and DNA nucleopeptide conjugates with many sequences.

Our experiments with DNA-catalyzed nucleopeptide formation have inherently addressed one important facet of peptide-modifying deoxyribozymes: their selectivity for the peptide substrate sequence. In principle, selecting for sequence *generality* is simple: one can alternate among various peptide sequences in a series of selection rounds, which directly imposes pressure on the emergent deoxyribozymes to function well with each of the consecutively presented peptide sequences. However, selecting for peptide sequence *selectivity* is a challenge. Frequently suggested is "negative selection" (also called "counterselection"), in which DNA sequences that catalyze an undesired reaction are actively removed from the population in some way. Unfortunately, negative selection is unlikely to be effective unless it is very stringent (because weak negative selection pressure still allows many undesirable sequences to survive), and developing a stringent negative selection against particular peptide sequences as deoxyribozyme substrates has not been possible. Fortunately, our experiments with azido-modified peptides directly provided a number of deoxyribozymes that were found to be peptide-sequence-selective despite the absence of any particular selection pressure for this outcome.[44] We suspect that the presence of various side chain functional groups on the peptide promoted the survival of DNA catalyst sequences that interact in particular ways with these side chains, which enforces the resulting peptide sequence selectivity.

A major and largely unaddressed challenge for all DNA-catalyzed side chain modification reactions, including nucleopeptide formation, is achieving their reactivity with large, intact

DOI: 10.1021/acs.accounts.5b00090
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protein substrates. We are currently pursuing a number of different approaches, whose full descriptions are beyond the scope of this Account. Obtaining peptide-sequence-selective deoxyribozymes is a component of this effort because such deoxyribozymes may recognize the corresponding "epitopes" when such sequences are presented as part of a larger protein substrate.

DEOXYRIBOZYMES WITH PHOSPHATASE AND KINASE ACTIVITIES

Given the important biological regulatory roles of phosphorylation, two substantial and biochemically relevant challenges for deoxyribozymes are DNA-catalyzed removal and attachment of phosphoryl groups from peptide side chains, i.e., phosphatase and kinase activities. We have identified deoxyribozymes with both kinds of activities, and in our current efforts we are seeking to expand their capabilities and applications.

Phosphomonoester hydrolysis is one of the slowest uncatalyzed reactions with biochemical relevance. The uncatalyzed half-life for phosphotyrosine (pTyr) hydrolysis is $\sim 10^4$ years, and the half-life for phosphoserine (pSer) hydrolysis is $\sim 10^{10}$ years. We developed an in vitro selection strategy to identify phosphatase deoxyribozymes that function on the time scale of hours (Figure 9). The key was the use of a capture reaction that itself requires a previously identified deoxyribozyme to attach an RNA strand specifically to Tyr but not pTyr, which leads to a PAGE shift for phosphatase deoxyribozymes (Figure 9A).[45] Using a suitable capture deoxyribozyme, we identified DNA-catalyzed phosphatase activity (Figure 9B).[46] These deoxyribozymes currently have the highest rate enhancement, $\sim 10^{14}$, of any known nucleic acid catalyst, primarily because the uncatalyzed rate constant is so small. Using the Zn^{2+}-dependent 14WM9 phosphatase deoxyribozyme, we demonstrated sequence-general tyrosine and serine phosphatase activity with a discrete peptide substrate, including when part of a 91-mer protein. In ongoing work, we are pursuing sequence-specific phosphatase activity and investigating DNA-catalyzed phosphatase activity inside human cells.

In parallel, we have sought DNA-catalyzed phosphorylation of peptide substrates, i.e., kinase activity.[47] We again used a capture deoxyribozyme, this time specific for attaching an RNA strand to pTyr rather than Tyr (i.e., the opposite preference of that depicted in Figure 9A). The phosphoryl donor was either a 5′-triphosphorylated RNA oligonucleotide (Figure 10A) or GTP. Deoxyribozymes were identified for tyrosine phosphorylation, including one with a K_m for GTP of merely ~ 20 μM (Figure 10B; many natural protein enzyme kinases have much worse [higher] K_M values for ATP as the phosphoryl donor). In unpublished work, we have found deoxyribozymes with serine kinase activity using 5′-triphosphorylated RNA as the phosphoryl donor. We are also exploring the use of binding to an immobilized phosphopeptide antibody rather than a PAGE shift as the selection basis. Current challenges that we are addressing for DNA-catalyzed kinase activity include achieving catalysis with discrete peptide substrates and peptide sequence selectivity.

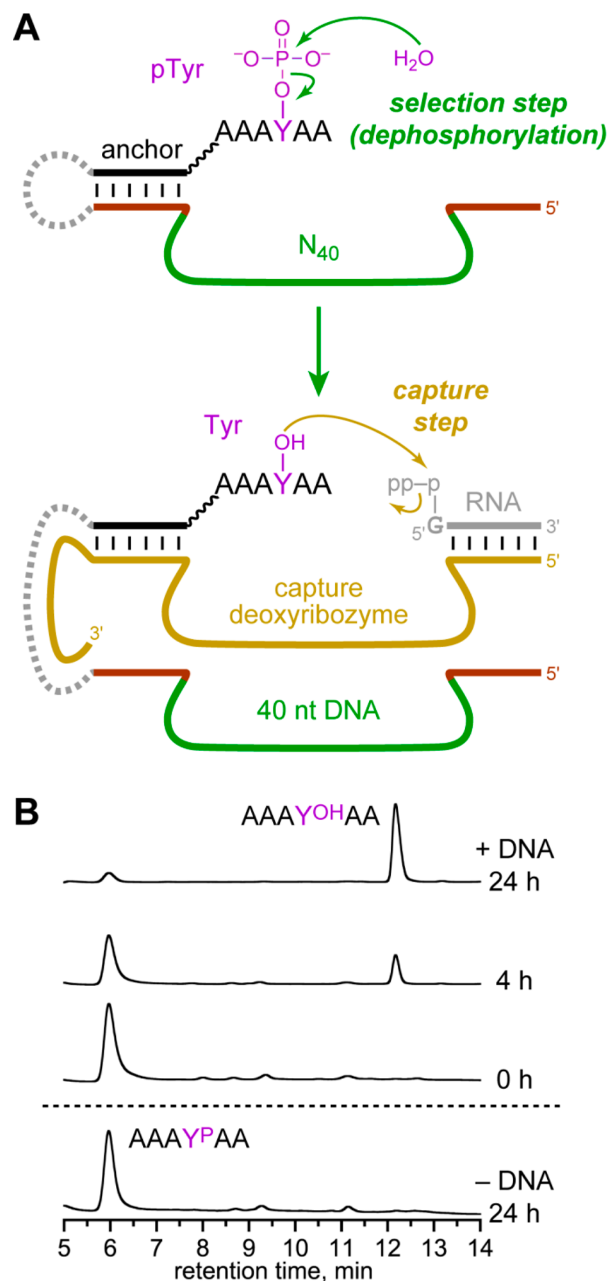

Figure 9. DNA-catalyzed peptide dephosphorylation (phosphatase activity).[45] (A) Selection strategy using a capture deoxyribozyme that is highly selective for Tyr over pTyr. (B) Multiple-turnover peptide dephosphorylation by the Zn^{2+}-dependent 14WM9 deoxyribozyme (500 μM peptide, 100 μM DNA).

DNA-CATALYZED LYSINE SIDE CHAIN MODIFICATION

The absence of DNA-catalyzed lysine reactivity in our first nucleopeptide study[37] induced us to consider more carefully why deoxyribozymes for reactions of nitrogen nucleophiles are so difficult to identify. When a more reactive 5′-phosphorimidazolide (Imp) electrophile was provided in place of the 5′-triphosphate (ppp), successful DNA catalysis with lysine was observed, even when the 3HJ architecture was abandoned in favor of the less preorganized arrangement (Figure 11).[48] This study was valuable for revealing the greater importance, at least in this context, of substrate reactivity relative to preorganization. These findings especially inform our ongoing efforts

DOI: 10.1021/acs.accounts.5b00090
Acc. Chem. Res. 2015, 48, 1369−1379

Figure 10. DNA-catalyzed peptide phosphorylation (kinase activity).[46] (A) Selection strategy. 5′-Triphosphorylated RNA is shown as the phosphoryl donor; in separate selections, GTP or ATP can be provided instead. (B) GTP concentration dependence of the 8EA101 (N_{30}) and 16EC103 (N_{50}) deoxyribozymes.

toward DNA-catalyzed amine (e.g., lysine) acylation reactions, in which we are evaluating a variety of reactive acyl donors.

EVALUATING THE IMPORTANCE OF THE RANDOM REGION LENGTH

For every in vitro selection experiment that starts with an entirely random pool, the investigator must predetermine the nucleotide length of that pool, i.e., the random region length. In others' work the pool has been as long as N_{228}, for which an amazingly miniscule fraction of sequence space (10^{-123}) is

Figure 11. DNA-catalyzed lysine reactivity using a reactive 5′-phosphorimidazolide (Imp) as the electrophile.[48]

explored. Two competing considerations inform the choice of random region length. On the one hand, smaller random regions allow greater coverage of sequence space (representative fractions are 10^{-10}, 10^{-22}, and 10^{-34} for N_{40}, N_{60}, and N_{80}, respectively), and also, the corresponding functional sequences are relatively unlikely to misfold or be inhibited by other, nonfunctional sequence segments. On the other hand, larger random regions allow greater complexity of secondary and tertiary structure, which may be beneficial or even necessary for certain functions.

We assessed experimentally the importance of the random region length to the selection outcome for two different DNA-catalyzed reactions, DNA cleavage and nucleopeptide formation.[49] The results revealed markedly different outcomes for the two reactions. For DNA cleavage, N_{20}, N_{30}, and N_{40} were successful whereas N_{50} and N_{60} were unsuccessful. In contrast, for nucleopeptide formation, N_{20} was unsuccessful whereas the longer random region lengths up to N_{60} were successful, and moreover, N_{40}-derived DNA catalysts were the most effective.

These quantitative outcomes cannot be extrapolated to predict the detailed results of unrelated selections. Nevertheless, this study provided direct experimental evidence—the first for deoxyribozymes—that the random region length is an important variable in the design of an in vitro selection procedure. Most laboratories appear to choose a single random region length for their selection experiments, suggesting that this consideration is not broadly appreciated in practice, no matter how well-acknowledged theoretically. After obtaining these results, we have routinely begun to examine various random region lengths as part of our selection designs, and we encourage others to do so as well.

MODULAR DEOXYRIBOZYMES FOR USE OF SMALL-MOLECULE SUBSTRATES

For some kinds of side chain modification reactions, a small-molecule substrate is required or is at least one straightforward option. For example, DNA-catalyzed phosphorylation requires a phosphoryl donor, which may be a nucleotide 5′-triphosphate such as GTP or ATP (see Figure 10). Starting from branched RNA formation in the 3HJ architecture, earlier we rationally engineered an NTP binding site into an existing deoxyribozyme, but doing so required considerable biochemical information.[50] We were interested to assess experimentally whether it would be helpful to provide a predetermined small-molecule binding site in the form of an aptamer, which would

DOI: 10.1021/acs.accounts.5b00090
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cooperate functionally with an initially random catalytic region from the outset of selection (Figure 12).[51] The selection outcome established that while modular deoxyribozymes that utilize a distinct predefined aptamer domain can indeed be identified, such DNA catalysts do not have any functional advantage relative to nonmodular analogues selected simultaneously for binding and catalysis, at least for our test case of tyrosine kinase activity using an ATP phosphoryl donor. A principal value of this result is its guidance of ongoing selection experiments in which small-molecule aptamers could conceivably be integrated. One particular example is DNA-catalyzed glycosylation. Given the experimental finding about modularity and function, we are focusing on direct selection of simultaneous glycosyl donor binding and glycosylation catalysis, rather than first identifying a DNA aptamer for a glycosyl donor and only then performing selection for glycosylation using a random catalytic region in the presence of this predefined aptamer domain.

OTHER DNA-CATALYZED REACTIONS UNDER DEVELOPMENT

We are pursuing a variety of other DNA-catalyzed reactions of peptide and protein substrates, whose full descriptions unfortunately do not fit within the space of this Account. These reactions include lysine acylation; formation of dehydroalanine (Dha) and dehydrobutyrine (Dhb) from phosphoserine (pSer) and phosphothreonine (pThr); site-specific PEGylation of tyrosine, serine, and lysine side chains; glycosylation; and glycosidase activity. In each of these cases, the reaction has a clear biochemical or biological value, such that a corresponding artificial DNA catalyst would have practical utility.

METAL ION COFACTORS, REACTION MECHANISMS, AND A CHOICE

This Account has not emphasized the important roles of metal ions in DNA catalysis. Others have exploited the metal ion selectivities of deoxyribozymes to create specific metal sensors, metal-dependent logic gates, or metal-dependent switches, often relying upon DNA-catalyzed RNA cleavage.[52−54] In our own work, we have used primarily the divalent metal ions Mg^{2+}, Mn^{2+}, and Zn^{2+}; we have also shown that trivalent lanthanide ions can be effective.[55] At present, our understanding of metal ion requirements is essentially phenomenological, which relates to the larger topic of reaction mechanisms. A deep understanding of DNA catalyst mechanisms will almost certainly require high-resolution X-ray or NMR structural information as a starting point (although combinatorial mutagenesis and biochemical studies can contribute[56−59]). To date, however, no such structural information is available for any deoxyribozyme.[60]

Therefore, someone interested right now in identifying new DNA catalyst function faces an important strategic choice: (1) devote substantial effort to structural biology of DNA catalysts, seeking to obtain enough information to enable rational design or at least rational redesign, or instead (2) focus on designing suitable selection strategies that allow identification of functional DNA sequences without requiring specific and detailed mechanistic information. Considering the nascent status of such design efforts with proteins,[7−9] despite tens of thousands of high-resolution protein enzyme structures combined with probably orders of magnitude more protein enzymology studies, my personal choice is to focus on DNA catalyst selection strategies rather than structural biology. This is not meant to downplay the value of structural biology. It merely recognizes that we are far from the time when structural understanding of DNA catalysts will enable their rational (re)design. With that in mind, my judgment is that new DNA catalyst function will be more fruitfully achieved at present by focusing on selection approaches.

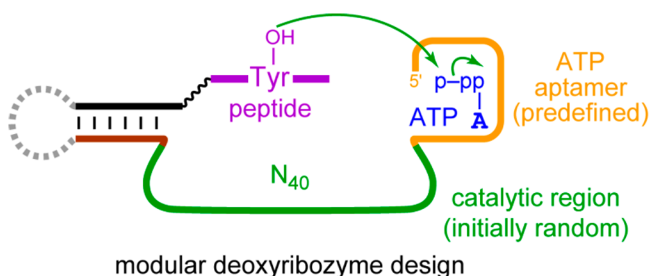

Figure 12. Selection design for identifying modular deoxyribozymes that integrate a distinct small-molecule binding site (aptamer) near an initially random catalytic region.[51]

PROSPECTS FOR DNA CATALYSTS

Our group has always strived to be cautious in predicting the ultimate value of deoxyribozymes, especially for potential practical applications. The growing range of established DNA-catalyzed reactions of peptide side chains, which includes such chemically very difficult transformations as pTyr/pSer dephosphorylation (phosphomonoester hydrolysis), suggests that deoxyribozymes are likely to have practical utility, especially after further development to address several challenges associated with protein substrates. These challenges include achieving useful catalytic parameters (k_{cat} and K_M) and selectivities among otherwise-identical side chains. If these hurdles can be overcome, which can be addressed only by continued experimentation, then deoxyribozymes identified by in vitro selection will have a valuable place alongside small-molecule catalysts and protein enzymes in the toolbox of practical catalysts.

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Notes

The author declares no competing financial interest.

Biography

Scott K. Silverman was born in 1972 and raised in Los Angeles, California. He received his B.S. degree in chemistry from UCLA in 1991, working with Christopher Foote on photooxygenation mechanisms. He was an NSF and ACS Organic Chemistry Predoctoral Fellow with Dennis Dougherty at Caltech, studying high-spin organic polyradicals and molecular neurobiology and graduating with a Ph.D. in chemistry in 1997. After postdoctoral research on RNA biochemistry as a Helen Hay Whitney Foundation and American Cancer Society Fellow with Thomas Cech at the University of Colorado at Boulder, he joined the University of Illinois at Urbana−Champaign in 2000, where he is currently Professor of Chemistry. His laboratory studies DNA as a catalyst.

DOI: 10.1021/acs.accounts.5b00090
Acc. Chem. Res. 2015, 48, 1369−1379

■ ACKNOWLEDGMENTS

I am grateful for the many outstanding contributions of the graduate students, undergraduates, and postdocs who have contributed to the research described here. This research has been supported primarily by the National Institutes of Health (NIGMS).

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