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Review

Biologically Inspired Synthetic Enzymes Made from DNA

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In cells, DNA typically consists of two antiparallel strands arranged in a double-helical structure, which is central to its fundamental role in storing and transmitting genetic information. In laboratories, however, DNA can be readily synthesized as a single-stranded polymer that can adopt many other types of structures, including some that have been shown to catalyze chemical transformations. These catalytic DNA molecules are commonly referred to as DNAzymes, or deoxyribozymes. Thus far, DNAzymes have not been found in cells, but hundreds of structural and functional variations have been created in the laboratory. This alternative catalytic platform has piqued the curiosity of many researchers, including those who seek to exploit them in potential applications ranging from analytical tools to therapeutic agents. In this review, we explore the unconventional role of DNA as a biologically inspired synthetic enzyme.

Introduction

Enzymes play an essential and pervasive role in biological systems, because they can act as highly efficient and selective catalysts under mild conditions. The general utility of natural enzymes has inspired the development of new synthetic versions (Jiang et al., 2008; Rothlisberger et al., 2008; Seelig and Szostak, 2007), which may eventually provide access to useful biotechnology, biomedicine, and industrial applications.

Although protein would be the most obvious choice of biomaterial from which to construct a synthetic enzyme, other types of biopolymers have also been investigated for this purpose. With fewer functional groups to assist in the formation of an active site, nucleic acids may seem like a poor alternative, and yet nucleic acid enzymes have a tangible and even fundamental presence in cells, as exemplified by the ribosome. This RNA-protein complex is responsible for protein synthesis in all three domains of life, and catalysis of the key peptidyl transfer step has been attributed to large ribosomal RNA (Cech, 2000; Nissen et al., 2000). Other naturally occurring ribozymes that catalyze the cleavage or formation of RNA phosphoesters have also been discovered (Doudna and Cech, 2002), and an even broader range of chemical reactions can be catalyzed by synthetic ribozymes (Silverman, 2009).

The concept of nucleic acid catalysis has been further validated by the demonstration that DNA also has the capacity for catalytic function. DNAzymes have not been found in nature, but they have been created in test tubes through a process known as in vitro selection (Baum and Silverman, 2008). The study of catalytic DNA can be rationalized from both an academic and practical perspective. Finding the ''lowest common denominator'' between different types of catalytic biopolymers may provide a better understanding of the fundamental principles that govern enzymatic catalysis. Furthermore, there are several notable advantages to using DNA over RNA or protein, which could be leveraged toward the practical application of DNAzymes. First, DNA is more stable to use than RNA and protein. The absence of a 2′-OH group at each phosphodiester linkage makes DNA ~100,000-fold more stable to hydrolysis than RNA under physiological conditions (Li and Breaker, 1999a). DNA phosphodiester bonds are also ~1000-fold more resistant to hydrolytic degradation than are peptide bonds (Smith and Hansen, 1998). Second, DNA is less expensive and can be easily prepared through solid-phase synthesis. These two preceding properties can generally make DNA more versatile and convenient to use. Other practical characteristics of DNA include the fact that it can be directly amplified by PCR (whereas RNA must first be reverse transcribed), it can function in solution and on surfaces via immobilization, and it can be readily chemically modified to increase stability or provide extra functional moieties. Therefore, DNAzymes can complement and extend the type of applications that may be suitable for synthetic enzymes. The various DNAzymes that have been reported to date should also help to dispel any preconceived notions about the functional limitations of DNA-based enzymes.

The Functional Repertoire of Synthetic DNA Enzymes

Enzyme active sites must precisely align catalytic and reacting groups to lower the activation-energy barrier of a given reaction. This requires a level of structural sophistication that the typical double-stranded DNA helix would not be able to offer. Therefore, it is important to recognize that single-stranded DNA can access many more types of secondary and tertiary interactions, such as nonstandard base pairs, hairpin loops, internal bulges, multistem junctions, pseudoknots, and stacked guanine quadruplex structures (Breaker, 1997). These additional interactions can contribute to the formation of more intricate structures consistent with the expectations of a catalytically active molecule. The demonstration that a tRNA and its DNA analog can adopt very similar structures was one of the early indications that single-stranded DNA had the capacity to form complex structures (Paquette et al., 1990). Furthermore, the demonstration that all but four positions within the hammerhead ribozyme could

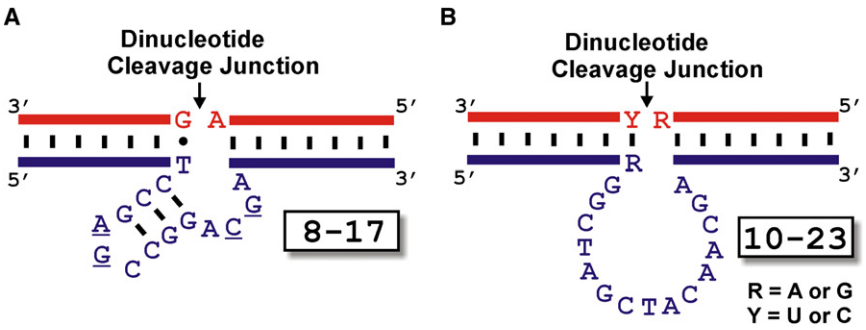

Figure 1. Sequence and Secondary Structures of the 8-17 and 10-23 RNA-Cleaving DNAzymes

The DNAzyme (blue) binds the RNA substrate (red) through Watson-Crick base pairing. For simplicity, only nucleotides that comprise the catalytic core of each DNAzyme are shown.

(A) 8-17. The catalytic core contains only four (underlined) highly conserved nucleotides that are required for robust activity. The original 8-17 sequence and cleavage site described by Santoro and Joyce (1997) is illustrated, but faster sequence variants with different cleavage site preferences have been identified (Schlosser et al., 2008b).

(B) 10-23. The catalytic core consists of 15 nucleotides, of which most are highly conserved.

be replaced with deoxyribonucleotides, without completely abolishing activity, suggested that DNA could indeed participate in the construction of catalytically competent structures (Perreault et al., 1990; Yang et al., 1992).

Any doubts about the catalytic ability of DNA were ultimately resolved in 1994, when the first DNAzyme was reported (Breaker and Joyce, 1994). This initial account described the isolation of a 38 nt single-stranded DNA molecule, which catalyzed the Pb^{2+}-dependent cleavage of an RNA phosphoester embedded within a separate DNA molecule. The cleavage reaction obeyed Michaelis-Menten kinetics with a multiple turnover k_{cat} value of 1 min^{-1} at 23°C and pH 7.0. The DNAzyme provided a rate enhancement of $\sim 10^5$-fold over the uncatalyzed reaction—a modest achievement by protein enzyme standards, but nevertheless an important step forward in the emerging field of DNA catalysis. Since then, many new DNAzymes have been reported, which serve to demonstrate the functional versatility of this alternative catalytic platform. These DNAzymes can emulate some of the same fundamental properties that are characteristics of traditional protein enzymes, including high rate enhancements, high specificity, and in some but not all cases, the ability to perform multiple substrate turnovers.

DNAzymes that catalyze the cleavage of RNA are by far the largest class of catalytic DNA molecules (Silverman, 2005), although the list of DNA-catalyzed reactions also include RNA ligation (including the formation of native 3′-5′ and nonnative 2′-5′ phosphodiester linkages) (Flynn-Charlebois et al., 2003a, 2003b; Purtha et al., 2005), RNA branching (Coppins and Silverman, 2005; Pratico et al., 2005; Wang and Silverman, 2003), RNA lariat formation (Wang and Silverman, 2005b), DNA depurination (Hobartner et al., 2007; Sheppard et al., 2000), oxidative DNA cleavage (Carmi et al., 1996, 1998; Carmi and Breaker, 2001), DNA ligation via 3′ or 5′ activation (Cuenoud and Szostak, 1995; Sreedhara et al., 2004), DNA branching (Mui and Silverman, 2008), DNA coupling (i.e., formation of a phosphorothioester linkage) (Levy and Ellington, 2001, 2002b), DNA phosphorylation (Li and Breaker, 1999b; McManus and Li, 2007; Wang et al., 2002), DNA adenylation (Li et al., 2000b), phosphoramidite cleavage (Burmeister et al., 1997), porphyrin metallation (Li and Sen, 1996), peroxidation (Travascio et al., 1998, 1999), thymine dimer cleavage (Chinnapen and Sen, 2004), formation of nucleopeptide linkages (Pradeepkumar et al., 2008), and formation of carbon-carbon bonds (i.e., through Diels-Alder cycloaddition) (Chandra and Silverman, 2008). The various DNAzyme-catalyzed reactions established to date have been reviewed in greater detail elsewhere (Achenbach et al., 2004; Baum and Silverman, 2008). The majority of reactions listed above involve the processing or modification of oligonucleotide substrates, primarily because such reactions are more amenable to the in vitro selection process by which DNAzymes are identified.

Rate enhancements vary by several orders of magnitude depending on the type of reaction, but so far the upper limit that has been observed is $\sim 10^{10}$-fold, for a DNAzyme that catalyzes DNA adenylation (Li et al., 2000b). It should be noted that although this represents the highest empirical value reported in the literature, it remains plausible that higher rate enhancements may still be achieved in the future.

Because of their significant applicative potential (as will be discussed later), RNA-cleaving DNAzymes have been the subject of numerous studies (Silverman, 2005). One of the most thoroughly investigated RNA-cleaving DNAzymes is a small metalloenzyme known as "8-17" (Santoro and Joyce, 1997). Not unlike protein enzymes, 8-17 can demonstrate good specificity at multiple levels—first at the substrate-binding step, and then at the chemical step of the transesterification reaction it catalyzes. As can be seen in Figure 1, 8-17 engages a cognate nucleic acid substrate by the formation of Watson-Crick base pairing upstream and downstream of the target cleavage site. One advantage of this type of interaction is that the sequence of the substrate-binding arms can be modified to recognize any desired substrate sequence, thereby providing a versatile mechanism for the targeted cleavage of RNA. In contrast, protein ribonucleases such as RNase T1 cleave indiscriminately at all locations containing G residues. The 8-17 DNAzyme also has a specific requirement for the identity of the two nucleotides flanking the cleavage site. In this case, 8-17 catalyzes the cleavage of a 5′-GG dinucleotide junction most effectively, whereas other junctions can be cleaved with different levels of reduced activity (Schlosser et al., 2008b). Finally, 8-17 shows regiospecificity by cleaving native 3′-5′ phosphodiester linkages but not unnatural 2′-5′ linkages (Wang and Silverman, 2005a). Enantioselectivity has also been demonstrated by other reported RNA-cleaving DNAzymes (Ordoukhanian and Joyce, 2002).

The catalytic efficiency (k_{cat}/K_M) of 8-17 under simulated physiological conditions (37°C, 3 mM Mg^{2+}, and pH 7.4) has been reported as 4×10^5 M^{-1} min^{-1} (Bonaccio et al., 2004). However, studies conducted on another small RNA-cleaving DNAzyme known as "10-23" (Figure 1) have revealed that it has a catalytic efficiency of 3.2×10^8 M^{-1} min^{-1} under similar conditions, and as high as 4.9×10^9 M^{-1} min^{-1} under elevated Mg^{2+}

concentrations (Santoro and Joyce, 1997, 1998). These values corresponded very closely to the measured rates of enzyme-substrate association (i.e., by the rate of DNA-RNA hybridization in this case), suggesting that 10-23 can achieve near-catalytic "perfection," consistent with the Albery and Knowles definition as a situation in which the reaction rate is limited only by the rate of diffusional encounter between enzyme and substrate (Albery and Knowles, 1976).

The folding characteristics of 8-17 have also been examined in some depth (Kim et al., 2007a, 2007b; Liu and Lu, 2002). These studies have provided evidence indicating that catalysis in 8-17 can be activated by either an "induced-fit" or a "lock-and-key" mechanism, depending on the type of divalent metal ion used. Thus, DNAzymes can exploit the same modes of activation that RNA and protein metalloenzymes use, providing further evidence of the fundamental similarities between all three catalytic platforms. If DNAzymes can use the same modus operandi as protein enzymes, it seems reasonable to suggest there may be fewer intrinsic limitations to proficient DNA catalysis than generally perceived.

Catalytic Strategies Employed by DNAzymes

DNA lacks the type of functional groups found on amino acid side chains that are most commonly recognized as catalytic residues, including the imidazole of His, the carboxylate of Asp and Glu, the alkyl amine of Lys, and the sulfhydryl of Cys. Furthermore, at neutral pH, the nucleobases of DNA do not have significant acid/base properties. Despite these apparent limitations, DNA has shown a remarkable aptitude for catalysis as described in the preceding section. Therefore, the basic principles that govern protein catalysis are likely to be applicable to nucleic acid catalysis as well, although the chemical differences between polypeptides and polynucleotides could make some catalytic strategies more accessible to one biopolymer over the other.

Most DNAzymes require one or more divalent metal ions for activity. DNA is likely predisposed to utilize this type of catalytic strategy because there are many potential binding/coordination sites for positively charged metal ions along the negatively charged phosphate backbone, as well as the N and O atoms of the nucleobases. Divalent metal ions can facilitate the folding of DNA into higher-order structures, and/or could potentially act as Lewis acids and general bases (in the form of a metal hydroxide) that contribute directly to chemical catalysis by stabilizing the attacking and/or leaving groups (Narlikar and Herschlag, 1997). It should be noted that in most cases the precise role of the obligate metal cofactor, structural and/or catalytic, has not been elucidated.

Other types of cofactors have also been used productively by DNAzymes. An RNA-cleaving DNAzyme was identified that utilized the amino acid histidine as a cofactor, for which the imidazole group was suggested to act as a general base (Roth and Breaker, 1998). A DNAzyme that catalyzes site-selective depurination with the aid of periodate (as an apparent oxidizing agent) has also been reported (Hobartner et al., 2007).

DNAzymes that can function in the absence of divalent metal cofactors have also been developed (Geyer and Sen, 1997, 2000). In one report, an RNA-cleaving DNAzyme that only required monovalent ions exhibited a rate enhancement of $\sim 10^8$-fold over the uncatalyzed reaction (Geyer and Sen, 1997). The authors speculated that the DNAzyme may use one or more of the following catalytic strategies: general acid or base catalysis, precise positioning of reactive groups, and/or stabilization of the transition state. It should be noted that acid/base catalysis has never been explicitly demonstrated in DNAzymes. However, it is conceivable that the folded structure of a DNAzyme could create a microenvironment in which the pKa of relevant nucleobases are perturbed to near-neutrality, thereby making them more suitable for acid/base catalysis. This scenario has been implicated in the mechanisms of various nucleolytic ribozymes (Bevilacqua and Yajima, 2006).

The DNAzyme field can still be considered to be in its formative years, as much of the finer mechanistic details have yet to be elucidated, or even adequately addressed. For instance, high-resolution structural data for any DNAzyme (in a productive conformation) have not been reported, although some apparent effort has been made toward this end (Nowakowski et al., 1999). Therefore, this aspect of the DNAzyme field should represent a priority for future research. In the meantime, it may be instructive to review the mechanisms employed by natural ribozymes (Cochrane and Strobel, 2008), which have been far more thoroughly investigated, and should provide relevant insight into the possible catalytic strategies employed by DNAzymes.

Functional Limitations of DNA-Mediated Catalysis: Can DNAzymes Compete with Ribozymes and Protein Enzymes?

The large variety of deoxyribozymes that have emerged over the last 15 years has lent credence to the concept of a DNA enzyme, yet there is still an underlying question as to the fundamental limits of DNAzyme-mediated catalysis. Does DNA have the capacity to match the catalytic proficiency observed with ribozymes or even protein enzymes? The fewer functional groups in DNA could arguably constrain the structural versatility of DNAzymes, and impose an intrinsic (albeit undefined) limit on the magnitude of any catalytic function relative to RNA or protein. However, there is growing empirical evidence to suggest that the functional disparity between DNAzymes and more traditional biocatalysts may actually be quite small (at least in some cases).

The absence of a 2′-hydroxyl group in DNA (which removes a potential hydrogen-bond donor/acceptor) does not appear to have significant functional consequences for deoxyribozymes relative to ribozymes. The rate enhancements between various types of DNAzymes and their synthetic ribozyme counterparts are generally comparable (Baum and Silverman, 2008). DNAzymes (Santoro and Joyce, 1997) and synthetic ribozymes (Tang and Breaker, 2000) that cleave RNA are a good example. Analogous rate enhancements have also been observed when direct comparisons have been experimentally attempted, as is the case with two different DNAzymes that were derived from the sequence of existing ribozymes that catalyze carbon-carbon bond formation (Chandra and Silverman, 2008) and RNA ligation (Paul et al., 2006).

In comparing the relative catalytic abilities of DNA and RNA, it may also be instructive to consider the minimum requirements under which each class of polymer can sustain activity. Ribozymes and deoxyribozymes have shown a similar level of tolerance for the use of simpler nucleotide alphabets which lack

one or more of the four standard nucleotides. For instance, an RNA ligase ribozyme was first evolved to function in the absence of cytidine (Rogers and Joyce, 1999), and subsequently in the absence of both cytidine and guanosine (Reader and Joyce, 2002). The resulting ribozyme was ~20,000-fold less active than the original parent molecule from which it was derived (containing all four types of ribonucleotides), but still ~36,000-fold faster than the uncatalyzed reaction. Similarly, an RNA-cleaving DNAzyme with a simplified catalytic core composed of just guanosine and cytidine nucleotides has also recently been reported (Schlosser and Li, 2009). This DNAzyme was ~10,000-fold less active than the parent DNAzyme from which it was derived (containing all four types of nucleotides), but still ~5,300-fold faster than the uncatalyzed reaction. Collectively, these empirical examples suggest that DNA can exhibit the same structural and functional plasticity as RNA, and indicate that differences in their base identity and backbone composition do not represent a significant functional limitation for one or the other.

The differences between DNA and protein are far more extensive, and expected to create a larger functional gap between them. Nevertheless, there are theories that challenge this perception, and even some experimental evidence that suggests the functional gap may not be insurmountable. A conceptual framework has been proposed to understand the current "speed limit" (i.e., a rate constant of ~2 min^{-1}) that is commonly observed among synthetic RNA-cleaving ribozymes and deoxyribozymes (Breaker et al., 2003). The authors of this study investigated the kinetic characteristics of a variety of synthetic ribozymes and deoxyribozymes, and concluded that they use only some combination of two of the four possible catalytic strategies that could accelerate this reaction. It follows that nucleic acid enzymes that can optimally exploit three or more strategies could potentially achieve protein-like rate enhancements. Consistent with this theory, some natural RNA-cleaving ribozymes can exceed the aforementioned speed limit by two to three orders of magnitude (Canny et al., 2004; Roychowdhury-Saha and Burke, 2006; Zamel et al., 2004) and approach the ~80,000 min^{-1} rate constant exhibited by the protein enzyme RNase A (del Cardayre and Raines, 1994). Presumably, the higher degree of structural complexity in these natural ribozymes (including the use of structural elements peripheral to the putative catalytic core) (Khvorova et al., 2003) can allow them to use extra catalytic strategies, and/or to use them more optimally as compared to their synthetic counterparts.

Although analogous rate constants for RNA-cleaving DNAzymes have yet to be experimentally demonstrated, there are other examples where the catalytic properties of DNAzymes have compared favorably with their protein counterparts. For instance, a porphyrin metallating deoxyribozyme exhibits a k_{cat} of 3.8×10^{-3} s^{-1} (Li and Sen, 1996), which is slightly higher than the k_{cat} of 2.4×10^{-3} s^{-1} exhibited by a corresponding catalytic antibody for the same reaction (Cochran and Schultz, 1990). The 10-23 deoxyribozyme cleaves RNA with a k_{cat}/K_M of ~10^9 M^{-1} min^{-1} under optimal conditions (Santoro and Joyce, 1997, 1998), which is ~10-fold higher than the analogous value for RNase A (del Cardayre and Raines, 1994). Of course, these select examples are not representative of the majority of DNAzymes identified to date, but they do raise a valid question: is the true catalytic power of DNAzymes limited by chemistry, or just by discovery? Whereas natural protein enzymes have had the benefit of billions of years of evolution to refine their catalytic abilities, all DNAzymes have been created through test-tube evolution experiments, which are typically conducted on a timescale measured in weeks or even days.

Searching Sequence Space through In Vitro Selection

The development of in vitro selection in 1990 (also referred to as SELEX when used to isolate molecular recognition elements called aptamers) (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990) provided a powerful new approach to directly explore the catalytic abilities of nucleic acid enzymes, by creating synthetic versions in a laboratory. In vitro selection is currently the only way by which DNAzymes can be created, as it is not yet practical to rationally design them from first principles.

Every in vitro selection experiment begins with the basic presumption that DNAzymes are present in random sequence space, and simply need to be found. The primary benefit of in vitro selection is that it can identify DNAzymes from hundreds of trillions of random sequences, without systematically screening every sequence for activity. The challenge is to devise a selection strategy that can effectively discriminate between the catalytically active versus inactive molecules that are typically in excess by many orders of magnitude.

An initial combinatorial library composed of ~10^{14} different DNA sequences (generated by solid-phase chemical synthesis using appropriate DNA phosphoramidate mixtures) has traditionally provided sufficient diversity to satisfy the requirement that at least one sequence (and often more) can adopt a functional structure. In order to distinguish between active and inactive molecules, the reaction they catalyze must result in some form of self-modification (e.g., a change in size or change in the ability to bind an immobilized reagent) that allows the active molecules to become "selectable." For instance, an RNA-cleaving deoxyribozyme can cleave a covalently linked RNA substrate and therefore become shorter in length. These active molecules can then be separated from the longer uncleaved (inactive) molecules by virtue of their increased mobility during gel electrophoresis (although this may seem to contradict the notion that a true catalyst accelerates the rate of a reaction without itself being consumed; in practice, it is usually possible to convert a self-modifying deoxyribozyme into a true catalyst, simply by separating the substrate and enzyme into distinct functional domains). The selected DNA molecules then serve as templates for amplification by the polymerase chain reaction (PCR). PCR amplification is possible because the DNA sequences in the original library are designed with primer-binding sites. Mutagenic PCR (Cadwell and Joyce, 1994) is sometimes used to intentionally introduce random mutations into the sequence population, in an effort to cover more sequence space.

Prior to the next round of selection, the double-stranded PCR product must be separated into individual strands so that the DNAzyme-coding strand is free to fold into its catalytically active structure. A variety of mechanisms can be used to this effect, as described elsewhere (Silverman, 2005). The overall process of in vitro selection constitutes a cycle that typically must be

repeated for multiple rounds, until the active molecules are sufficiently represented to enable their identification by molecular cloning and sequencing. Depending on the specific number and type of steps involved in a given selection scheme, one round of selection can potentially be completed in a single day, and anywhere from 5 to 15 rounds of selection may be required in total. Multiple rounds of in vitro selection are always required, because some fraction of inactive "background" sequences will have a finite probability of surviving each round of selection simply by chance.

In essence, DNAzyme engineers design selection schemes to partition the catalytically active molecules away from the inactive molecules, and with each round of selective amplification, the ratio of active to inactive molecules in the pool is effectively increased. Ultimately, by progressively increasing the selection stringency over successive rounds (e.g., by decreasing the selection time or cofactor concentration), the many trillions of random sequences in the original library will be narrowed down to one or more sequences that can best meet the selection criteria (Figure 2). A more comprehensive description of the in vitro selection process has been provided elsewhere (Silverman, 2008).

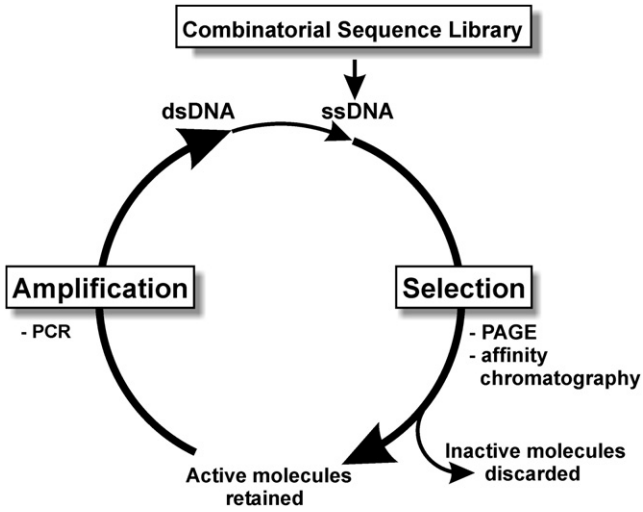

Figure 2. Conceptual Diagram of In Vitro Selection
In vitro selection begins with a combinatorial DNA library typically composed of $\sim 10^{14}$ different sequences. Individual molecules are selected for a desired activity, and the active molecules are partitioned away from the inactive molecules, usually through polyacrylamide gel electrophoresis (PAGE) or some form of affinity chromatography. The active molecules are then amplified by the polymerase chain reaction (PCR). The resulting double-stranded DNA molecules are converted into single-stranded DNA molecules (i.e., by removal of the noncoding antisense strand), and then used to initiate the next round of selective amplification.

Recognizing the Limits of In Vitro Selection and the Implications for Mapping the Functional Limits of DNAzymes

Sequence space can be defined as all the sequence permutations (or some subset) associated with an oligonucleotide of N random nucleotides. Because DNA is composed of only four different nucleotides, the total sequence space represented by a DNA molecule composed of N random nucleotides is 4^N sequences. There exists an enormous disparity between the amount of sequence space that is theoretically accessible (e.g., $\sim 10^{30}$ different sequences for N = 50) versus the amount that can be practically searched in a single experiment (i.e., $\sim 10^{14}$ molecules). To put these numbers in more physical terms, it would take more than 30,000 tons of DNA to screen all possible sequence permutations represented by just 50 random nucleotides. In contrast, in vitro selection experiments are limited to microgram quantities of DNA, and therefore can only sample a minuscule fraction of the total available sequence space.

If sequence space cannot be explored exhaustively, mapping the limits of DNAzyme function will ultimately depend on how *effectively* it can be explored. Toward this end, efforts to understand how the outcome of in vitro selection can be influenced by different experimental parameters such as the reaction time (Schlosser and Li, 2004), type of divalent metal cofactors (Wang et al., 2002), length of the random sequence domain (Gevertz et al., 2005; Sabeti et al., 1997), excess sequence elements (Schlosser et al., 2006), and population dynamics (Schlosser and Li, 2005) will continue to be a constructive pursuit. These studies can provide insight on how best to access new functional motifs, and avoid the recurrence of existing catalytic molecules such as 8-17, for which variants have been isolated on at least six separate occasions (Cruz et al., 2004; Faulhammer and Famulok, 1996; Li et al., 2000a; Peracchi, 2000; Santoro and Joyce, 1997; Schlosser et al., 2008a; Schlosser and Li, 2004).

Along the same lines, the development of techniques that can remove some of the constraints associated with standard in vitro selection strategies will be highly useful. In vitro selection experiments that incorporate automation (Eulberg et al., 2005), recombination (Bittker et al., 2002), continuous evolution (Johns and Joyce, 2005), and/or multiple turnover (e.g., through in vitro compartmentalization) (Levy et al., 2005) selection formats have provided new ways to facilitate sequence space exploration for ribozymes and aptamers. These techniques may not be immediately applicable to DNAzymes, but they represent the kind of innovations that could potentially be developed to benefit the DNAzyme field as well.

DNAzymes for Diverse Applications In Vitro and In Vivo

Aside from the inherent academic interest in DNAzyme-mediated catalysis, research in this field is also motivated by the potential practical applications. These applications can be broadly classified into several categories including molecular biology tools, therapeutic agents, nanomotors, analytical tools, and computational devices. Table 1 provides some perspective on the breadth of applications in which DNAzymes have participated. Some of these applications are of immediate practical utility (such as the use of RNA-cleaving DNAzymes as in vitro endoribonucleases), whereas others have been validated in proof-of-principle studies, but must still overcome additional obstacles before they can be used productively (such as DNA computational devices). The majority of these applications utilize 8-17 and 10-23 described earlier (Figure 1), because they possess several practical characteristics including (1) small size (i.e., $\sim$30 nt), (2) relatively high activity and multiple turnover abilities (i.e., $\sim 1\ min^{-1}$, but can be faster or slower depending on buffer conditions), (3) the ability to cleave both all-RNA and chimeric substrates (i.e., DNA oligonucleotides containing an embedded ribonucleotide), (4) the ability to function with

Table 1. Applications of DNAzymes In Vitro and In Vivo

Primary Category	Secondary Category	Examples	Selective References
Molecular biology tools	Practical RNA cleavage		Pyle et al., 2000
	Practical RNA ligation	3′-5′ linkages	Purtha et al., 2005
		Lariat RNA	Wang and Silverman, 2005b
		Branched RNA	Pratico and Silverman, 2007; Pratico et al., 2005; Wang and Silverman, 2006
	RNA labeling		Baum and Silverman, 2007
Therapeutic agents: mRNA cleavage in vivo	Antiviral	Influenza A	Takahashi et al., 2004
		Hepatitis C core protein	Trepanier et al., 2006
		HIV-1	Chakraborti and Banerjea, 2003
	Antibacterial	β-lactamase	Hou et al., 2007b
		penicillin-binding protein	Hou et al., 2007a
	Anticancer	VEGFR2	Zhang et al., 2002
		c-Jun	Fahmy et al., 2006; Zhang et al., 2004
		Egr-1	Bhindi et al., 2006; Mitchell et al., 2004; Santiago et al., 1999
Nanomotors: mechanical motion	Opening/closing		Chen and Mao, 2004; Chen et al., 2004
	Walking		Tian et al., 2005
Analytical tools: Detection and/or quantitation	Proteins	Streptavidin	Stojanovic et al., 2000
		Lysozyme	Li et al., 2007
	Nucleic acids	DNA and/or RNA	Sando et al., 2003; Tian and Mao, 2005; Todd et al., 2000
		RNA	Suenaga et al., 2005
	Sequence polymorphisms		Cairns et al., 2000; Cairns and Sun, 2004
	RNA modifications	2′-O-methylribose	Buchhaupt et al., 2007
		pseudouridines	Buchhaupt et al., 2007; Hengesbach et al., 2008
		m^5C	Hengesbach et al., 2008
	Small molecules	Adenosine	Liu and Lu, 2004a
		AMP	Li et al., 2007
		ATP	Cho et al., 2005; Levy and Ellington, 2002a; Mei et al., 2003
	Metal ions	Pb^{2+}	Chang et al., 2005; Li and Lu, 2000; Liu and Lu, 2003a; Xiao et al., 2007
		UO_2^{2+}	Liu et al., 2007
		Cu^{2+}	Liu and Lu, 2007a, 2007b
		Hg^{2+}	Hollenstein et al., 2008; Liu and Lu, 2007c
Computational devices	Boolean function logic gates		Chen et al., 2006; Stojanovic et al., 2002, 2005
	Arithmetic operations	Half-adder	Stojanovic and Stefanovic, 2003a
		Full adder	Lederman et al., 2006
	Automata	Circuits that play tic-tac-toe	Macdonald et al., 2006; Stojanovic and Stefanovic, 2003b

A representative overview of the various types of applications in which DNAzymes have been employed. Some applications are of immediate practical utility, whereas others may represent proof-of-principle demonstrations of potential utility. The majority of these applications exploit the 8-17 and 10-23 RNA-cleaving DNAzymes (or sequence variations thereof).

different divalent metal cofactors, and (5) versatile substrate recognition abilities (i.e., substrate recognition arms that can be modified to match virtually any target sequence). These characteristics have contributed to their widespread and convenient integration into different types of applications. It is beyond the scope of this review to address the full applicative potential of DNAzymes; however, we will briefly highlight a few applications of particular significance or interest.

The in vitro manipulation of RNA represents the most obvious and straightforward application of DNAzymes that catalyze RNA cleavage (Silverman, 2005). DNAzyme-based endoribonucleases can offer an immediate practical alternative to

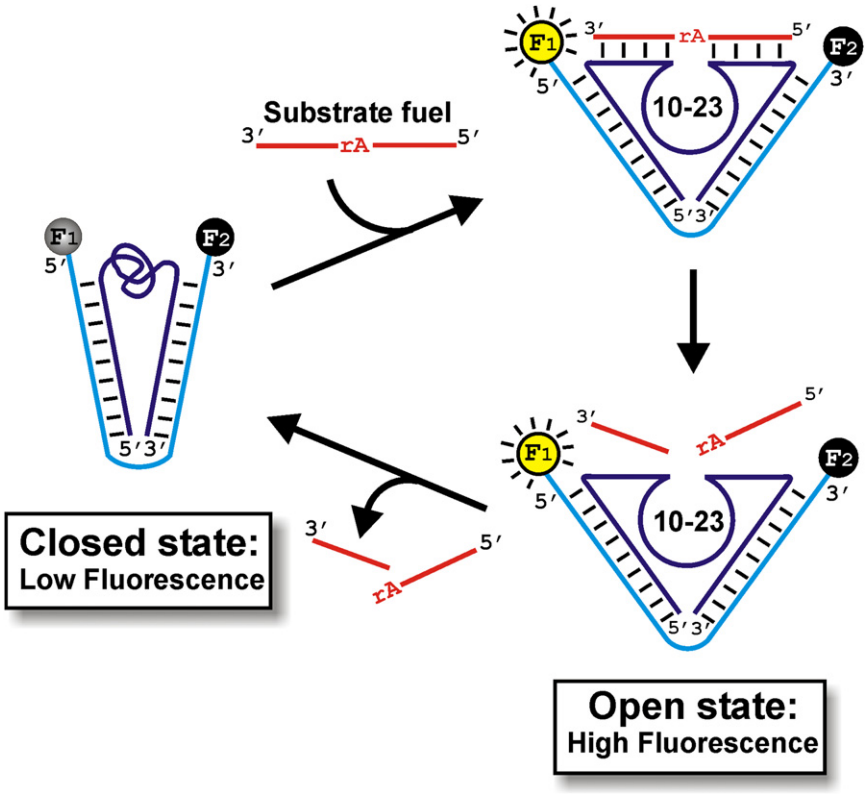

Figure 3. A DNA Nanomotor Based on 10-23
The DNA motor is composed of two oligonucleotide strands. One strand (blue) contains 10-23, and the other strand (cyan) contains a donor-acceptor pair of fluorophores, F1 and F2, on opposing ends. The presence of the two fluorophores allows any change in motion to be observed through fluorescence resonance energy transfer (FRET). The motor is fueled by a chimeric (DNA/RNA) oligonucleotide substrate (red), which is susceptible to cleavage by 10-23. In the absence of the substrate, the motor adopts a "closed" conformation, characterized by a low fluorescence signal. However, when the substrate hybridizes to the DNAzyme, the DNA motor adopts an "open" conformation that leads to an increased fluorescence signal. Cleavage and subsequent dissociation of the substrate allows the DNA motor to once again adopt the closed conformation. This motor continues to cycle between the open and closed states as long as substrate is available.

existing ribozyme (Ferre-D'Amare and Doudna, 1996) and RNase H (Lapham and Crothers, 1996) techniques for the preparation of homogeneous RNA transcripts, and for the general processing activities involved in RNA structural studies (Pyle et al., 2000). The utility of RNA-cleaving DNAzymes can also be extended to in vivo applications. In this respect, they have been investigated as tools to dissect the biological function and/or to downregulate the expression of genes associated with disease. A growing number of studies have demonstrated the potential for these DNAzymes to selectively attenuate the expression of various antibacterial (Hou et al., 2007a, 2007b), antiviral (Chakraborti and Banerjea, 2003; Takahashi et al., 2004; Trepanier et al., 2006), and anticancer targets (Fahmy et al., 2003; Khachigian et al., 2002; Mitchell et al., 2004; Zhang et al., 2002, 2004). Their efficacy has been evaluated in cell-culture assays as well as in several relevant animal models of cancer, in which tumor sizes were reduced by 60%–90% (Fahmy et al., 2003; Khachigian et al., 2002; Mitchell et al., 2004; Zhang et al., 2002, 2004). For a more comprehensive description of the various therapeutic targets of DNAzymes, readers are encouraged to consult any one of several good reviews (Bhindi et al., 2007; Dass et al., 2008; Peracchi, 2004).

10-23 has been the most common choice for in vivo applications of RNA-cleaving DNAzymes, largely because it was the first DNAzyme to be thoroughly characterized, and shown to sustain a relatively useful level of activity under the low Mg^{2+} concentrations found in cells. Unfortunately, 10-23 has a specific requirement for a 5′-purine-pyrimidine dinucleotide cleavage site, which can impose some restrictions on its utility. Therefore, the identification of new DNAzymes that can separately, or in combination, enable the efficient cleavage of all 16 possible RNA dinucleotide junctions under physiological conditions represents an important ongoing challenge. Thus far, it has proven difficult to satisfy all of these requirements. For instance, a combination of in vitro selection and mutagenesis studies has led to the identification of different 8-17 sequence variations (Cruz et al., 2004; Schlosser et al., 2008b) and completely new DNAzymes (Schlosser et al., 2008a) that collectively cleave all 16 dinucleotide junctions with a minimum rate constant of ~0.1 min^{-1}. However, efficient cleavage was generally only observed in the presence of Mn^{2+}, and against chimeric substrates (which are often used because they can provide a specific cleavage site during in vitro selection). Although improvements to the activity and cleavage site versatility of RNA-cleaving DNAzymes are expected to facilitate their use and efficacy in vivo, there are additional factors that may ultimately limit their utility. As with other nucleic acid-based therapeutic strategies such as antisense, ribozyme, and siRNA, the practical implementation of DNAzymes can be complicated by issues related to delivery, intracellular stability, cellular distribution, and off-target effects. For a discussion of the relative merits of these competing strategies, the reader is referred elsewhere (Baum and Silverman, 2008; Bhindi et al., 2007; Peracchi, 2004).

DNAzymes have also been incorporated into more avant-garde applications that attempt to mimic aspects of biological systems. For instance, DNAzymes have been used to power autonomous DNA nanomotors that can perform continuous conformational changes without human interference (Figure 3). Not unlike cellular protein motors that employ an ATPase to extract chemical energy from ATP, these DNA nanomotors use an RNA-cleaving DNAzyme to extract the chemical energy stored in RNA phosphodiester bonds. This chemical energy is subsequently converted into mechanical motions that imitate the opening and closing action of scissors (Chen and Mao, 2004; Chen et al., 2004), or even walking (Tian et al., 2005).

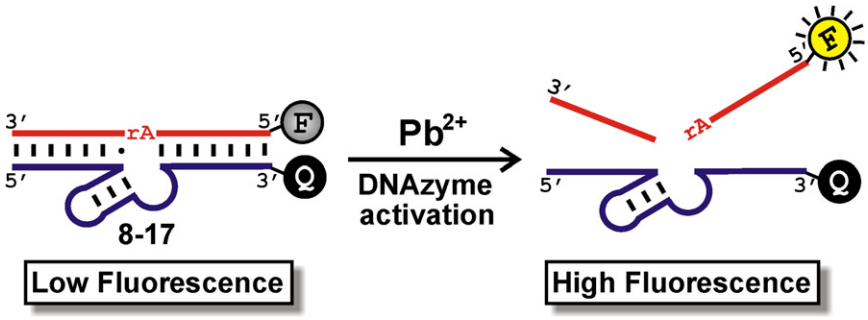

Figure 4. A Pb^{2+} Biosensor Based on 8-17
The 5′ end of the substrate and the 3′ end of 8-17 are labeled with a fluorophore (F) and quencher (Q), respectively. The DNAzyme (blue) binds to the substrate (red) through conventional Watson-Crick base pairing (vertical lines), which positions the quencher close to the fluorophore to limit fluorescence emission. In the presence of Pb^{2+} ions, the DNAzyme becomes active and cleaves the single ribonucleotide linkage (rA, adenosine ribonucleotide) embedded in an otherwise all-DNA oligonucleotide. The individual cleavage fragments have lower affinity for the DNAzyme, and therefore dissociate to yield a concomitant increase in fluorescence intensity as the fluorophore moves away from the quencher.

Various analytical devices have also been developed from DNAzymes, in order to detect nucleic acids, metal ions, and small organic molecules. One of the first DNAzyme biosensors was based on 8-17, and exploited its Pb^{2+}-dependent RNA cleavage activity (Figure 4) (Li and Lu, 2000). A Pb^{2+} biosensor was constructed with a fluorescent reporting system, by simply labeling the 5′ end of the substrate oligonucleotide with a fluorophore, and the 3′ end of the DNAzyme strand with a fluorescence quencher. In the uncleaved state, the substrate binds to the DNAzyme, positioning the fluorophore and quencher in proximity to each other for maximal fluorescence quenching. When Pb^{2+} is introduced into the solution, the DNAzyme becomes active and cleaves the substrate, which subsequently dissociates from the DNAzyme to generate a fluorescence signal. The signaling properties of this original design have been improved by modifying the number, arrangement, and type of fluorophores and quenchers used (Chiuman and Li, 2007; Liu and Lu, 2003b). Alternative reporter systems have also been developed based on colorimetric (Liu and Lu, 2003a, 2004b) and electrochemical detection methods (Xiao et al., 2007). Biosensors for other types of toxic metal ions including Cu^{2+} (Liu and Lu, 2007a, 2007b), Hg^{2+} (Hollenstein et al., 2008; Liu and Lu, 2007c), and UO_2^{2+} (Liu et al., 2007) have been developed as well. These biosensors can offer exquisite sensitivity and selectivity, as reflected by the UO_2^{2+} sensor that exhibits parts per trillion sensitivity and million-fold selectivity (Liu et al., 2007).

The utility of DNAzyme biosensors can be further expanded by creating allosterically regulated versions. Although the concept of allosteric control in nucleic acid enzymes has been explored more extensively for ribozymes (Breaker, 2002), representative examples of allosteric deoxyribozymes that respond to small molecules such as adenosine and ATP have also been reported (Cho et al., 2005; Levy and Ellington, 2002a; Liu and Lu, 2004a; Mei et al., 2003; Shen et al., 2006). Allosteric DNAzymes incorporate an aptamer domain, which provides additional molecular recognition abilities. Aptamers are single-stranded DNA (or RNA) molecules that can bind with high affinity and specificity to many different types of molecules including metal ions, small organic compounds, metabolites, and proteins (Famulok et al., 2000). The activity of an allosteric DNA enzyme is regulated by the ligand-binding state of the aptamer, through a ''communication module'' (Breaker, 2002) that is typically a weakened stem structure. Ligand binding causes a conformational change in the aptamer domain that leads to stabilization of both the communication module and DNAzyme, with a concomitant increase in the catalytic activity. Allosteric DNAzymes based on this modular design philosophy can provide a versatile platform for broad target recognition in diagnostic applications.

The amenability of DNAzymes to rationally designed allosteric control has also been exploited to create molecular-scale computational elements. For instance, allosteric RNA-cleaving DNAzymes that respond to the presence and/or absence of one or more oligonucleotide effectors with the production of a signal have been engineered into logic gates that can execute Boolean functions such as ''AND,'' ''NOT,'' and ''XOR'' (Stojanovic et al., 2002). The potential intricacy of control that can be exerted by DNAzyme-based logic gates has been demonstrated through the development of higher-order systems that employ networks of parallel logic gates to perform arithmetic operations (Lederman et al., 2006; Stojanovic and Stefanovic, 2003a), or even function as automata to play games such as tic-tac-toe (Macdonald et al., 2006; Stojanovic and Stefanovic, 2003b). In addition, logic gates constructed from different types of DNAzymes have been constructed to increase their versatility. For instance, DNAzymes that catalyze DNA ligation were used to generate larger output molecules that could subsequently serve as input molecules for downstream gates (Stojanovic et al., 2005), thereby demonstrating the potential for signal transduction cascades, by utilizing a combination of both parallel and serial circuits. Of course, these DNA-based computational devices are not expected to compete with silicon-based computers. Instead, because of their biocompatibility, DNAzyme logic gates have been envisaged as a cellular platform from which to control autonomous therapeutic and/or diagnostic devices (Stojanovic et al., 2002). For example, DNAzyme logic gates could potentially analyze the presence of molecular disease markers and make appropriate decisions to trigger the release of a drug.

Conclusions

Synthetic DNA molecules have the capacity to catalyze chemical transformations with high activity and selectivity, and therefore can potentially offer a practical alternative to more traditional biocatalysts. The use of these DNAzymes, alongside RNA and protein enzymes, will no doubt enrich the scale and complexity to which researchers will be able to probe and manipulate the function of biological systems in the future. In the meantime, DNAzymes are currently more suitable for applications in vitro,

but their precise roles will likely be redefined as new and improved versions are identified.

Understanding what the true scope of DNA's catalytic abilities may entail is an intriguing question. However, a clear answer may never be found, because only small fractions of sequence space are actually subject to scrutiny. Nevertheless, every new in vitro selection experiment can provide additional clues to the global distribution of function in sequence space, and mapping the functional limits of DNA catalysis will depend, to a large extent, on how frequently and effectively this technique is employed. Of course, there are limits to what DNAzyme engineers alone can achieve. Significant progress in the DNAzyme field, and the productive use of its related applications, will ultimately be coupled to the development of complementary technologies in various other fields, including those at the interface of chemistry and biology.

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