

MNAzymes, a Versatile New Class of Nucleic Acid Enzymes That Can Function as Biosensors and Molecular Switches

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Abstract: To increase the versatility and utility of nucleic acid enzymes, we developed multicomponent complexes, known as MNAzymes, which produce amplified “output” signals in response to specific “input” signals. Multiple oligonucleotide partzymes assemble into active MNAzymes only in the presence of an input assembly facilitator such as a target nucleic acid. Once formed, MNAzymes catalytically modify a generic substrate, generating an amplified output signal that heralds the presence of the target while leaving the target intact. We demonstrated several applications including sensitive, isothermal target detection; discrimination of polymorphisms; and highly specific monitoring of real-time polymerase chain reaction (PCR). Furthermore, we showed their capacity to function as molecular switches and to work in series to create a molecular cascade. The modular nature of MNAzymes, together with the separation of input and output functionalities, provides potential for their integration into diverse devices such as diagnostic biosensors, molecular computers, and/or nanoscale machines.

Introduction

The extraordinary physical and functional properties of DNA and RNA have led to extensive investigation into their suitability for use in many fields including therapeutics, diagnostics, chemistry, nanotechnology, and molecular computation (reviewed in refs 1 and 2). In pursuit of these endeavors, researchers exploit both natural and contrived properties of nucleic acids, including interstrand and intrastrand hybridization, strand displacement, binding of ligands to aptamers, and catalysis by nucleic acid enzymes.

In general, nucleic acid enzymes are composed of single strands of either DNA (deoxyribozymes or DNAzymes) or RNA (ribozymes) that are organized into domains required for enzymatic activity (catalytic core domains) and for substrate recognition (substrate binding domains). DNAzymes and ribozymes are capable of catalyzing a broad range of chemical reactions including cleavage, ligation, phosphorylation, and deglycosylation of RNA or DNA (reviewed in ref 3). Catalytic nucleic acids, sometimes in combination with aptamers, have been studied extensively for their capacity to provide the basis for biosensors (reviewed in refs 1–5). Many strategies use nucleic acid sequences that include an intact catalytic core but have reduced enzymatic potential due to weakened association

of the enzyme with its substrate^{6,7} (reviewed in ref 4). In such designs, the presence of the target analyte induces a conformation change that increases substrate binding affinity and catalytic activity. For example, the target may disrupt an inhibitory hairpin structure⁶ or it may bind to the enzyme and substrate, thus strengthening their interaction.⁷ The disadvantage of this approach lies in the potential for low-level catalytic activity in the absence of the target analyte under low stringency conditions. An alternative and inherently safer strategy involves dividing the catalytic core between multiple oligonucleotides that are capable of combining to form active enzymes. Such oligonucleotides have been discovered by extensive re-engineering or in vitro evolution of ribozymes (reviewed in refs 8 and 9) or by rational design utilizing specific tertiary structures within DNAzymes such as guanine quartets¹⁰ or stem-loops.¹¹ For example, Kolpashchikov¹¹ chose the E6 DNAzyme for splitting because it contained a nonconserved stem-loop that was thought to be required for structural, but not catalytic, function.

In this report we describe a general approach for deriving partial catalytic core sequences from nucleic acid enzymes, which can then be incorporated into partial enzymes (partzymes) useful for assembly into MNAzymes. In contrast to previous studies, our strategy does not necessarily require knowledge of,

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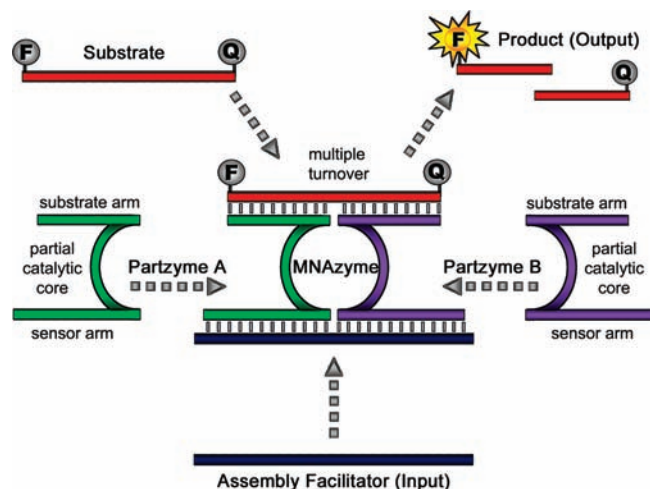


Figure 1. Schematic of an exemplary MNAzyme with cleavage activity. This MNAzyme was composed of partzymes A and B, each of which contained a substrate arm, a partial catalytic core, and a sensor arm that can bind to an assembly facilitator, such as a target nucleic acid molecule. The assembly facilitator provided the “input” that directed the assembly of partzymes into an MNAzyme with a functional catalytic core capable of cleaving multiple substrates into products thus providing an “output”. If the substrate is labeled on each side of the cleavage site with a fluorophore (F) and a matched quencher (Q), then cleavage can be monitored by an increase in fluorescence.

or exploitation of, predicted or observed structural or functional domains. We demonstrate our approach by engineering multiple partzyme pairs incorporating various sequences derived by splitting catalytic domains from two different nucleic acid enzymes, known as the 10–23 and 8–17 DNazymes.¹² While each partzyme is inherently inactive, when they are combined in the presence of an assembly facilitator, they may associate and form a catalytically active, multicomponent nucleic acid enzyme (MNAzyme). MNAzymes have numerous applications, including use as biosensors, as exemplified below where the target nucleic acids to be detected served as the assembly facilitators. However, assembly facilitators may also be synthetic and/or have multiple components, as shown in examples where MNAzymes allowed the creation of molecular switches and cascades. It is the modular nature of MNAzymes that imparts the enormous flexibility and functionality, which in turn increases the versatility of nucleic acid enzymes.

Results

Structure and Function of Multicomponent Nucleic Acid Enzymes (MNAzymes). MNAzymes are multicomponent nucleic acid enzymes that are assembled, and therefore catalytically active, only in the presence of an assembly facilitator (Figure 1). These enzymes are composed of multiple partial enzymes, or partzymes, which self-assemble in the presence of assembly facilitators and form active MNAzymes that catalytically modify substrates to produce products. Figure 1 provides an example of the components of an assembled MNAzyme capable of recognizing and cleaving a substrate in the presence of an assembly facilitator. The partzymes have multiple domains including sensor arms that bind to the assembly facilitator or target; substrate arms that bind the substrate; and partial catalytic core sequences that, upon assembly, combine to provide a complete catalytic core (Figure 1). MNAzymes can be designed

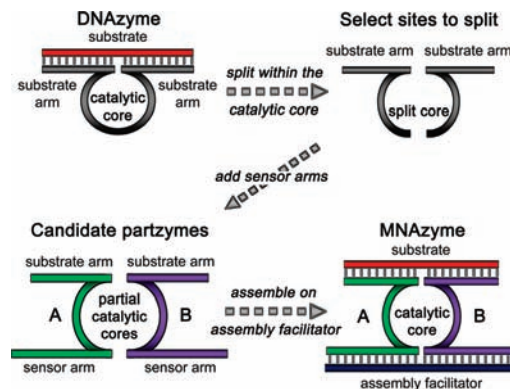


Figure 2. Engineering new MNAzymes from DNazymes. Many DNazymes have a catalytic core flanked by substrate binding arms. To engineer MNAzymes, sites for splitting the catalytic core of the DNzyme are selected, and then candidate partzymes A and B incorporating sensor arms are designed. Candidate partzyme pairs A and B may be tested by mixing them with a substrate and assembly facilitator and then assessing the level of catalytic activity.

to recognize a broad range of input assembly facilitators including, for example, different target nucleic acid sequences. In response to the presence of the assembly facilitator, MNAzymes modify their substrates, thus generating an enzymatically amplified output signal. The assembly facilitator may be a target nucleic acid present in a biological or environmental sample. In such cases, the detection of MNAzyme activity is indicative of the presence of the target in the sample.

The partial catalytic core modules in the following examples were engineered by splitting the catalytic core of the unimolecular DNA enzymes known as the 10–23 and the 8–17 DNazymes.¹²

Engineering MNAzymes from DNazymes. The general strategy we developed to engineer MNAzymes from DNazymes is illustrated in Figure 2. Many DNazymes have similar structures: a catalytic core flanked by substrate arms that can be tailored to hybridize to a range of substrates. To engineer a new MNAzyme from a DNzyme, positions are identified within the DNzyme catalytic core at which it can be split, so that each partial portion of the catalytic core can be distributed between two oligonucleotides (candidate partzymes A and B). Partzymes are designed such that the sequence from 5' to 3' of candidate partzyme A contains (i) one sensor arm portion capable of binding to an assembly facilitator molecule, (ii) one partial catalytic core portion derived from the DNzyme, and (iii) one substrate binding arm portion; and candidate partzyme B contains (i) one substrate binding arm portion capable of binding adjacent to candidate partzyme A on the same substrate sequence, (ii) one partial catalytic core portion that contains the remaining bases from the catalytic core of the DNzyme that were not incorporated into the candidate partzyme A, and (iii) one sensor arm portion capable of binding to the same assembly facilitator as candidate partzyme A and in a position adjacent to partzyme A (Figure 2).

A series of paired A and B candidate partzymes can be screened to determine whether they exhibit catalytic activity. Candidate partzyme pairs are assessed by mixing them with suitable substrates and assembly facilitators and then ascertaining the degree of catalytic activity, if any. Once active partzyme pairs have been identified, their partial catalytic cores can be incorporated into a range of new partzymes, which can be tailored for recognition of other assembly facilitators (e.g., new targets) and/or other substrates.

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Table 1. Kinetic Analysis Comparing 10–23 DNAzyme and Candidate Partzyme Pairs^a

DNAzyme	sequence of the complete catalytic core domain		k_{obs} , min ⁻¹
10–23	G ₁ G ₂ C ₃ T ₄ A ₅ G ₆ C ₇ T ₈ A ₉ C ₁₀ A ₁₁ A ₁₂ C ₁₃ G ₁₄ A ₁₅		4.0
candidate partzyme pair core	partial catalytic sequences in candidate partzyme pairs		k_{obs} , ^b min ⁻¹
	partzyme B (G ₁ to N _y)	partzyme A (N _x to A ₁₅)	
Mz1a	G ₁ to A ₅	G ₆ to A ₁₅	9.3×10^{-3}
Mz1b	G ₁ to G ₆	C ₇ to A ₁₅	1.9×10^{-3}
Mz1c	G₁ to C₇	T₈ to A₁₅	7.1×10^{-1}
Mz1d	G₁ to T₈	A₉ to A₁₅	1.2
Mz1e	G ₁ to A ₉	C ₁₀ to A ₁₅	1.1×10^{-1}
Mz1f	G ₁ to C ₁₀	A ₁₁ to A ₁₅	1.7×10^{-3}
Mz1g	G ₁ to A ₁₁	A ₁₂ to A ₁₅	4.6×10^{-3}

^a Partzyme pair core sequences based on the 10–23 DNAzyme are designated Mz1, with candidate partzyme sequences designated a–g. N represents a DNA base, and *x* and *y* denote the positions with respect to the complete catalytic core of the DNAzyme that are split upon incorporation into candidate partzymes A and B, respectively. ^b k_{obs} values were obtained by fitting a hyperbolic function (by use of Prism Version 4.0, GraphPad Software Inc.) to the fluorescence versus time data (Figure S1, Supporting Information).

The protocol for engineering MNAzymes from DNAzymes is illustrated in the following example where partzymes, based on the 10–23 DNAzyme,¹² were engineered and used as components for MNAzymes, referred to here as type Mz1. The sequence of the complete catalytic core domain of this DNAzyme is shown in Table 1. A control 10–23 DNAzyme (Dz1), which was capable of cleaving a chimeric DNA/RNA substrate designated Sub2i-FB, was synthesized. Substrate Sub2i-FB was labeled with a fluorophore (FAM) and quencher (Black Hole Quencher) on either side of the cleavage site. The 10–23 catalytic core was then split at a number of positions (Table 1) and candidate partzyme pairs were synthesized such that candidate partzyme B contained bases from G₁ to the split position, and candidate partzyme A contained bases from the split position to A₁₅. Candidate partzymes were designed to hybridize to both an assembly facilitator DNA oligonucleotide, AF-RO5, and the substrate, Sub2i-FB (Table S1, Supporting Information). Partzyme pairs were incubated in the presence of AF-RO5 and Sub2i-FB with 50 mM MgCl₂ and the change in fluorescence, indicative of cleavage of the substrate, was monitored over time to provide a measurement of k_{obs} (Figure S1, Supporting Information).

The results (Table 1; Figure S1, Supporting Information) indicated that the most suitable sites for splitting the 10–23 catalytic core to generate useful partial core pairs were between C₇ and T₈ (Mz1c) and between T₈ and A₉ (Mz1d). Partzymes based on splitting the core between A₉ and C₁₀ (Mz1e) showed activity, albeit reduced compared to the Mz1c and Mz1d splits, and other candidate pairs showed little activity. The k_{obs} values for control 10–23 DNAzyme and for Mz1c and Mz1d partzyme designs were comparable.

To confirm that the MNAzyme cleaves the substrate in a similar fashion to the original DNAzyme, Figure 3A shows a polyacrylamide gel demonstrating cleavage of the radiolabeled DNA/RNA chimeric substrate Sub2-FB by both a 10–23 DNAzyme and an MNAzyme (Mz2c) engineered as described above.

To demonstrate the generality of our approach for engineering MNAzymes from DNAzymes, the process was repeated and additional MNAzymes based on variants of the 8–17

DNAzyme¹² were produced; they are designated here as Mz2 MNAzymes (Tables S2 and S3, Supporting Information). Similar to Mz1 MNAzymes, the activity of Mz2 MNAzymes was influenced by the location of the core split (Figure S2, Supporting Information).

To further analyze MNAzyme activity, partzymes were synthesized to contain partial catalytic core sequences based on the 8–17 DNAzyme variant 1 (Mz2a), along with substrate arms complementary to a substrate (from ref 13), referred to here as Sub1i-FB. Incubation of partzymes A and B in the presence of the complementary assembly facilitator AF-RO4 resulted in the formation of active Mz2a MNAzymes that cleaved Sub1i-FB, resulting in an increase in fluorescence over time (Figure 3B). Control reactions, which lacked either partzyme B (control 1) or partzyme A (control 2) or which lacked the assembly facilitator and had an irrelevant noncomplementary oligonucleotide (control 4), did not show an increase in fluorescence. This confirmed that the increase in fluorescence was due to catalytic activity of the MNAzymes and was dependent on the hybridization and assembly of the partzymes with the assembly facilitator and that the partzymes, either alone or as a pair, had no catalytic activity until they were assembled into MNAzymes on the assembly facilitator.

To further confirm that the mechanism for generation of fluorescence was cleavage of Sub1i-FB by the MNAzymes, a C to T mutation was introduced into the partial catalytic core within an Mz2a partzyme A. This base is the equivalent of the C nucleotide at position 11 within the complete catalytic core domain and has been reported to be crucial for enzymatic activity of the 8–17 DNAzyme.¹⁴ When this mutation was incorporated into partzyme A, the catalytic activity of the MNAzyme was abolished (control 3, Figure 3B), consistent with a similar mechanism of catalysis between the DNAzyme and MNAzymes derived from it. Finally, the lack of any activity of the oligonucleotide complexes containing this mutated partzyme confirms that the increase in fluorescence generated by the active Mz2a MNAzymes is indeed due to cleavage of the substrate and is not due to a “beacon”-like effect caused by hybridization of the partzyme substrate-binding arms to the substrate.

Kinetics of MNAzyme Catalysis. The kinetics of MNAzymes were analyzed, with the Mz1d core split as an example, and compared to those of the 10–23 DNAzyme on which the Mz1 series was based. Both enzymes had identical substrate binding arm sequences and were designed to cleave substrate Sub2i-FB. Turnover was compared by measuring k_{cat} , that is, the amount of substrate converted to product per unit of time. Kinetic studies measured k_{cat} and K_{m} values for the MNAzyme as approximately 28 min⁻¹ and 95 nM, respectively. In comparison, the k_{cat} and K_{m} of the DNAzyme were approximately 80 min⁻¹ and 140 nM, respectively. $k_{\text{cat}}/K_{\text{m}}$ was 2.9×10^8 min⁻¹ M⁻¹ for the MNAzyme and 5.6×10^8 min⁻¹ M⁻¹ for the DNAzyme. Together these data demonstrate that an MNAzyme can function as a true enzyme capable of multiple turnover with only slightly decreased efficiency compared to the DNAzyme on which it is based.

Further, similar to DNAzymes, the rate of cleavage by MNAzymes was shown to be dependent on many factors including temperature, length of the substrate binding arms, and the constituents and concentration of buffer. The arm length of

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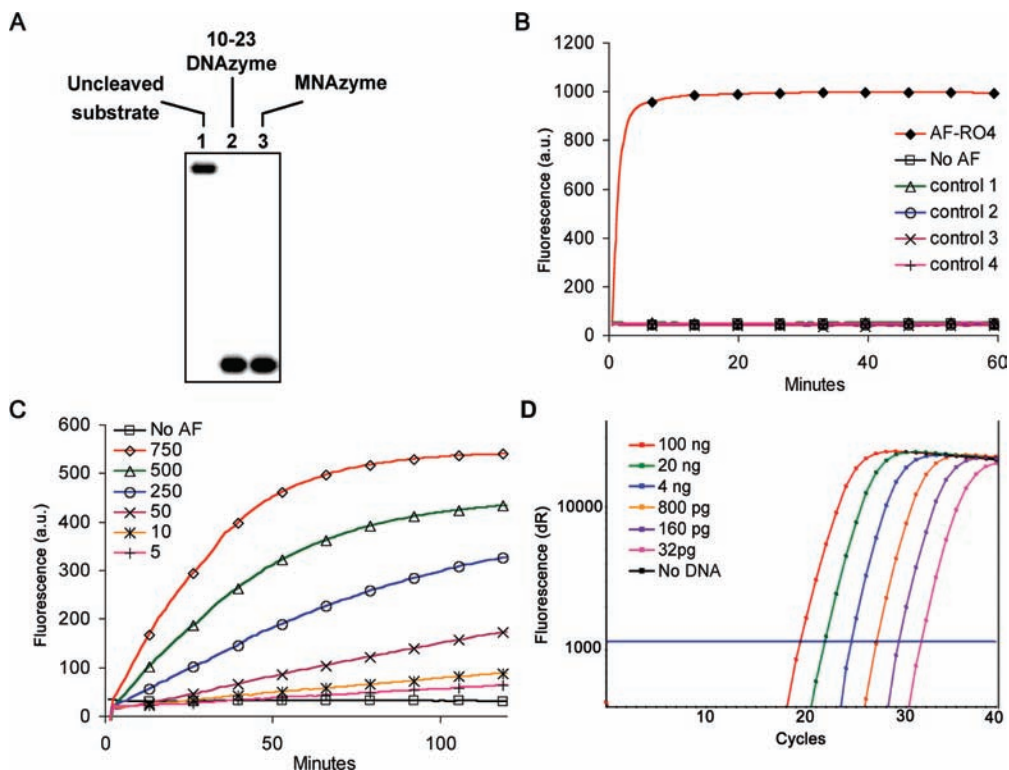


Figure 3. Cleavage of radiolabeled and fluorescent substrates by catalytic nucleic acids and use of MNzymes in the detection of target nucleic acids. (A) Polyacrylamide gel electrophoresis showing uncleaved radiolabeled substrate (lane 1) and cleavage products of this substrate following incubation with a DNAzyme (lane 2) and an MNzyme (lane 3). (B) Demonstration of cleavage activity by Mz2 MNzymes. Test reactions contained partzyme A (RO4Aa/1) and partzyme B (RO4Ba/1) in the presence of AF-RO4 or in the absence of an assembly facilitator (no AF). Control reactions contained partzyme A only with AF-RO4 (control 1); partzyme B only with AF-RO4 (control 2); mutated partzyme A with partzyme B and AF-RO4 (control 3); or partzymes A and B with a noncomplementary oligonucleotide AF-RO5 (control 4). (C) Use of Mz1d MNzymes to determine the limit of detection of a target assembly facilitator by the isothermal direct detection protocol. Test reactions contained RO3Ad/2-P, RO3Bd/2-P, and Sub2i-FB, with either various concentrations of target AF-RO3 (750, 500, 250, 50, 10, or 5 pM) or no assembly facilitator (no AF). (D) MNzymes were used to monitor the accumulation of amplicons during PCR amplification of the K-ras gene from human genomic DNA (5-fold dilutions from 100 ng to 32 pg). The calibration curve generated from this plot had an $R^2 = 0.999$ and an efficiency of 94%.

various substrates was optimized to allow cleavage by MNzymes under various conditions, including those compatible with real-time polymerase chain reaction (PCR). By way of example, measurement of k_{obs} of the substrate Sub2i-FB over a broad temperature range showed an optimum at around 50 °C (Figure S3, Supporting Information). In this example the number of bases in the substrate binding arms was 11 and 10 for the A and B partzymes, respectively. Analysis of other MNzymes showed that the optimal temperature decreased as the lengths of substrate arms decreased, consistent with faster product dissociation of shorter oligonucleotides at lower temperatures (data not shown). Santoro and Joyce¹² demonstrated that the rate of catalysis of the 10–23 DNAzyme is also dependent upon the concentration of divalent cations. Additional experiments confirmed that the rate of catalysis by this MNzyme was similarly dependent on the concentration of Mg^{2+} . The catalytic rate of this MNzyme increased approximately linearly with increasing concentrations over the range of 0–100 mM Mg^{2+} , after which the rate reached a plateau (data not shown).

Use of MNzymes for Detection and/or Quantification of Nucleic Acids. MNzymes can be used as tools for direct detection of target nucleic acids in a protein-free, isothermal, fluorescent format. The limit of detection of such an assay was assessed in a series of three experiments with Mz1d partzymes, which were designed to assemble in the presence of the target AF-RO3 and cleave Sub2i-FB. At 52 °C the MNzyme assay

could discriminate 5 pM of target assembly facilitator from the background signal (measured by the absence of target) (Figure 3C). This corresponds to approximately 125 amol or 7.5×10^7 copies of the target nucleic acid sequence in a 25 μL reaction.

MNzymes can also be used to provide the readout for real-time PCR. Mz1d MNzymes were designed to assemble in the presence of amplicons produced during PCR. Changes in fluorescence following cleavage of Sub2i-FB allowed real-time detection and quantification of the K-ras gene from human genomic DNA (Figure 3D). In the absence of genomic DNA, fluorescence did not increase. A standard curve was generated by plotting the log of the initial DNA concentration of a serial dilution of genomic DNA against the threshold cycle, resulting in a linear plot with a correlation coefficient of 0.999 and a PCR efficiency of 94%. Signal was detected in reactions containing as little as 32 pg of genomic DNA, corresponding to approximately 10 copies of the target gene, demonstrating the high sensitivity of MNzyme detection coupled with real-time PCR.

Discrimination of Single Base Differences by Use of MNzymes. Many variations of the basic MNzyme design, some of which involve using additional component oligonucleotides beyond those illustrated in Figure 1, have been shown to form active MNzymes (Figure 4) and provide further functionalities and utility. One possible variation incorporates a partzyme with a truncated sensor arm that requires hybridization of a stabilizer arm adjacent to it on the target to facilitate

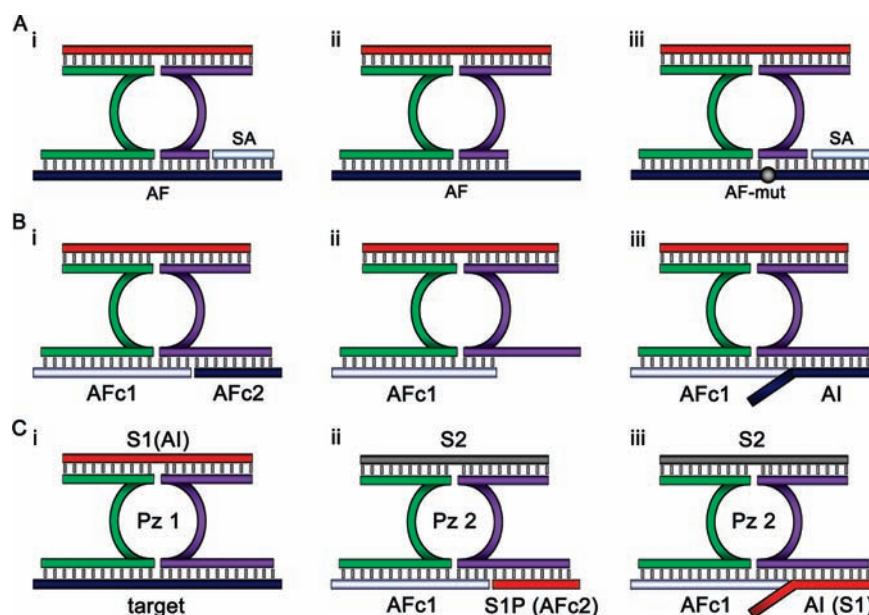


Figure 4. Structures used to produce molecular switches and molecular cascades. The following abbreviations indicate oligonucleotides in this figure: SA, stabilizer arm; AF, assembly facilitator; AF-mut, mutated assembly facilitator; AFc, assembly facilitator component; AI, assembly inhibitor; S, substrate; Pz, partzyme pair; S1P, cleavage product of substrate 1. AF-mut indicates a sequence that is identical to AF with the exception of a single base difference. Numbers are used to indicate the presence of more than one of any of the above elements in a particular series of complexes indicated in this figure. Some oligonucleotides can function in dual capacities. For example, the substrate S1 can also function as an AI and in such cases the second function is indicated in brackets. Similarly, once S1 is cleaved, one of its products (S1P) can function as AFc2. While some oligonucleotide complexes represent active MNAzymes (A i, B i, and C i and ii), others are catalytically inactive (A ii and iii, B ii and iii, and C iii).

formation of an active MNAzyme (Figure 4A i). This type of MNAzyme structure can be exploited to discriminate two targets differing by a single base (Figure 5A). In the following example, the sensor arm of one partzyme was truncated and contained only five bases, all of which were fully complementary to an assembly facilitator (AF-XdC). A second oligonucleotide (AF-XdT) was identical to AF-XdC with the exception that a thymine replaced a cytosine, thus resulting in a single mismatch with respect to the truncated partzyme sensor arm. A stabilizer arm, fully complementary to the adjacent sequence of both AF-XdC and AF-XdT, was added to the reaction. In the presence of the partzymes, the stabilizer arm, and the fully matched target AF-XdC, MNAzyme activity resulted in cleavage of the substrate Sub2i-FB (Figures 4A i and 5A i). Omission of the stabilizer arm from this reaction resulted in no cleavage, demonstrating this oligonucleotide was essential for formation of active MNAzymes incorporating the partzyme with the truncated sensor arm (Figures 4A ii and 5A ii). When the partzymes and the stabilizer arm were incubated with the mismatched target AF-XdT, no MNAzyme activity was evident, consistent with the capacity of MNAzymes to discriminate between two sequences differing by only a single base (Figures 4A iii and 5A iii). Together these data demonstrate that active MNAzymes, which incorporate partzymes with truncated sensor arms, are formed only when the stabilizer arm is present and the assembly facilitator is fully matched to the sensor arms. Absence of the stabilizer arm and/or the presence of a mismatch in the assembly facilitator within the region that hybridizes to the truncated sensor arm results in an inactive complex.

Use of MNAzymes To Create Molecular Switches and Cascades. MNAzymes can also be designed so that the formation of an active enzyme depends upon the presence of multiple assembly facilitator components (AFc), any one of which can be used to switch the enzyme from the “on” state (active MNAzyme) to an “off” state (inactive complex) or vice versa.

Further, inclusion of assembly inhibitor oligonucleotides can result in formation of alternative inactive complexes that incorporate and sequester MNAzyme components, including partzymes. These strategies allow the design of MNAzyme cascade reactions and molecular switches.

Structures incorporating two assembly facilitator components, AFc1-RO1 and AFc2-RO1, were shown to facilitate active MNAzyme formation followed by cleavage of the substrate Sub2i-FB and a resultant increase in fluorescence (Figures 4B i and 5B i). Omission of one of the assembly facilitators, AFc2-RO1, from this reaction resulted in inactive complexes (Figures 4B ii and 5B ii). An assembly inhibitor oligonucleotide (AI) was tested that incorporated one domain equivalent to the sequence of one assembly facilitator component (AFc2-RO1) plus a second domain containing noncomplementary bases appended to the termini at the junction with the other assembly facilitator component (AFc1-RO1) (Figure 4B iii). Incubation of the partzymes, one assembly facilitator component (AFc1-RO1), and the assembly inhibitor oligonucleotide (Sub6) again produced inactive complexes that were unable to cleave the substrate (Figure 5B iii). These alternate structures could function as a molecular switch that is “on” when AFc2-RO1 completes the formation of an active MNAzyme and is “off” when the assembly inhibitor binds in its place, forming an inactive complex.

Two MNAzymes were engineered to work in concert to produce a molecular cascade. Reactions were set up to contain partzymes and a substrate for both an “initiating” MNAzyme (RO5Ad/6-P, RO5Bd/6-P and Sub6-TRB) and a “cascading” MNAzyme (RO1Ad/2-P, RO1Bd/2-P, and Sub2i-FB) plus a first assembly facilitator component, AFc1-RO1, required for the active state for the cascading MNAzyme. Reactions either contained or lacked the target, AF-RO5. In the presence of the target, the initiating MNAzyme was formed, which cleaved the first substrate Sub6-TRB, resulting in an increase in Texas Red

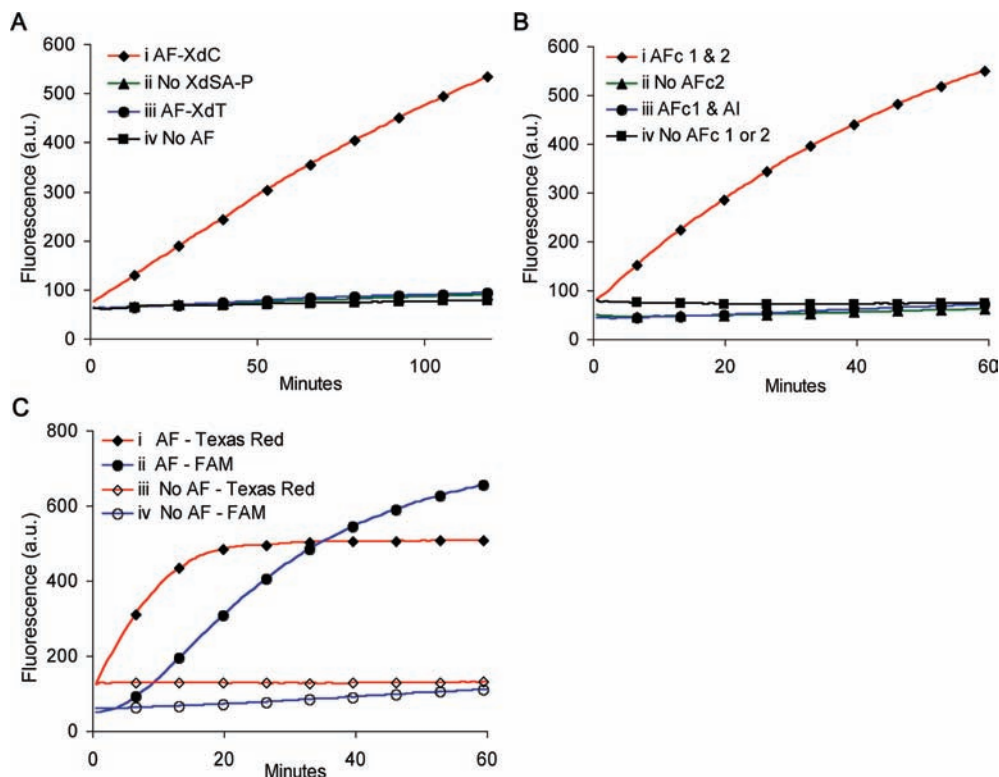


Figure 5. Analysis of activities of various combinations of structures shown in Figure 4. (A) Detection of a single base difference by use of a partzyme, XdBd/2-P, that has a truncated five-base sensor arm in combination with the stabilizer arm, XdSA-P. All reactions contained XdAd/2-P, XdBd/2-P, and Sub2i-FB. Reactions also contained XdSA-P in the presence of either (i) fully matched target (AF-XdC), (iii) mismatched target (AF-XdT), or (iv) no assembly facilitator. An additional control reaction (ii) contained fully matched target (AF-XdC) but lacked the stabilizer arm (XdSA-P). (B) Demonstration of active MNazymes requiring two assembly facilitator components and inactive complexes incorporating inhibitor sequences. All reactions contained RO1Ad/2-P, RO1Bd/2-P, and Sub2i-FB. In addition, reactions also contained (i) assembly facilitator components AFc1-RO1 and AFc2-RO1, (ii) no AFc2-RO1, (iii) AFc1-RO1 and assembly inhibitor AI (Sub6-TRB), or (iv) no assembly facilitator components. (C) Demonstration of MNazyme cascade reaction. Reactions contained the components of the initiating MNazyme (RO5Ad/6-P, RO5Bd/6-P, and Sub6-TRB) and the cascading MNazyme (RO1Ad/2-P, RO1Bd/2-P, Sub2i-FB, and AFc1-RO1). In addition, reactions either contained target AF-RO5 (i, ii) or lacked target (iii, iv), and fluorescence of either Texas Red (i, iii) or FAM (ii, iv) was monitored over time.

fluorescence (Figures 4C i and 5C i). Cleavage of Sub6-TRB by the initiating MNazyme produces a cleavage product (S1P) that is one of two facilitator components required to direct assembly of the cascading MNazyme. The cascading MNazyme then cleaved the second substrate Sub2i-FB, causing an increase in the fluorescence of FAM (Figures 4C ii and 5C ii). As such, an increase in fluorescence of both Texas Red and FAM is indicative of the presence of the target AF-RO5 in the reaction. In the absence of target sequence, no significant increase in fluorescence of either Texas Red or FAM was observed over time (Figure 5C iii and iv). In its intact state, uncleaved Sub6-TRB would bind to one of the partzymes of the cascading MNazyme and function as an assembly inhibitor oligonucleotide directing formation of inactive complexes (schematic in Figure 4C iii).

Discussion

This paper describes MNazymes, which are a powerful type of nucleic acid enzyme, with increased versatility and functionality that stems from the multiple components that make up these modular enzymes. The specific MNazymes characterized here are DNA enzymes that comprise partial enzymes or partzymes, each of which lacks part of the sequence necessary for catalysis. Partzymes combine to form catalytically active enzymes only in the presence of one or more assembly facilitators. Active MNazymes catalyze substrate modification, thus producing an output signal. When the assembly facilitator

is a target nucleic acid, modification of the substrate produces a signal that heralds the presence of the target in the sample.

Kinetic analysis established that MNazymes are capable of multiple turnover: for each target molecule present, multiple substrate molecules are modified and this results in signal amplification. The k_{cat} values observed for both the DNAzyme and MNazyme in this study are on the order of 10-fold higher than the maximum k_{cat} reported for a DNAzyme by Santoro and Joyce.¹² Several factors, such as the use of higher pH and temperature, may have contributed to this result; however, the most significant contributor is likely due to differences in the composition of the substrates used in the studies. Santoro and Joyce¹² observed that k_{cat} was highly dependent on the specific base composition of the substrate, and Ota et al.¹⁵ demonstrated that a specific DNAzyme cleaved a DNA/RNA chimeric substrate with an enhanced rate compared to its all RNA counterpart. The substrate used in kinetic studies by Santoro and Joyce was an in vitro-transcribed RNA substrate selected because it corresponded to the start codon within the HIV target. In contrast, our kinetic studies used a DNA/RNA chimeric substrate that was selected by screening hundreds of different substrate sequences for those that afforded faster turnover rates. A subset of substrates was chosen as the basis for a series of

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generic substrates, each of which can be incorporated into single or multiplexed MNAzymes assays for detection of a broad range of targets.

A general strategy for engineering new MNAzymes based on DNAzymes was outlined here. We demonstrated the generality of this approach by deriving MNAzymes from 10–23 and 8–17 DNAzymes.¹² MNAzymes were found to have similar properties to the DNAzymes from which they were engineered. For example, these MNAzymes were shown to be true enzymes capable of multiple turnover at a rate that is dependent on both the concentration of magnesium ions and the reaction temperature. Like protein enzymes, DNAzyme catalysis is highly dependent on tertiary structure formation, and factors that impact this even subtly can have a profound influence on the catalytic rate.^{15–17} Therefore, it is remarkable that MNAzymes have enzyme activity comparable to that of DNAzymes when their fundamental differences in structure are considered. This is particularly true for MNAzymes based on the 10–23 DNAzyme, which has no apparent stem–loop or other tertiary structures within the catalytic core. Such structures could theoretically promote the formation of an active MNAzyme catalytic core by supporting association and/or stabilization of partial catalytic domains when the complete DNAzyme core has been split between two partzymes. For example, the presence of the structural stem–loop within the parent E6 DNAzyme most likely contributes to the association of component oligonucleotides that constitute the binary deoxyribozyme developed by Kolpashchikov.¹¹ While the enzymes exemplified in our paper all cleave nucleic acid substrates, recent work in our laboratory has extended this repertoire by deriving an MNAzyme capable of ligation (data not shown). Furthermore, the strategy described here could be applied to derive new MNAzymes from either DNAzymes or ribozymes, which could have a broad range of catalytic functions.

Much of the power of MNAzymes comes from the modularity imparted by component partzymes. In practice, partzymes can be synthesized to have sensor arms that hybridize to any target nucleic acid of interest and substrate arms that hybridize to any one of a series of generic substrates. Examples in this report have used various combinations that incorporated seven partial core sequence pairs, five target binding sequences, and three substrate binding sequences to produce 12 different MNAzymes. Further, the use of generic substrates as probes provides multiple advantages regardless of the application for which MNAzymes are being used. In particular, the use of generic substrates in protocols allowing target detection confers reliable, consistent performance regardless of the target sequence. Further, their use reduces assay development time, lowers the cost of goods, and eliminates substrate waste since any excess can be used for the next system.

MNAzymes have functionalities useful for a range of biotechnical applications, especially molecular diagnostics, where demand is increasing for point-of-care assays and technologies with higher throughput, sensitivity, and specificity. In two demonstrations of utility described here, MNAzymes have enabled detection and/or quantification of target nucleic acids. We reported the use of MNAzymes in an isothermal, protein-free assay for direct detection of nucleic acids. The assay detected approximately 125 amol of target in 2 h by fluorescence monitoring in a format compatible with analysis on any general-

purpose fluorometer. The ability of this MNAzyme to detect a low concentration of target was superior to that reported for the E6 binary deoxyribozyme,¹¹ which only detected 1 nM after 30 h. Our MNAzyme detection strategy provides a simple, inexpensive protocol suitable for detecting targets present at a high copy number in a point-of-care setting. This isothermal MNAzyme protocol could also produce a colorimetric readout if combined with an approach such as that published by Lui and Lu.¹⁸ However, methods that use either DNAzymes or split DNAzymes to produce a colorimetric readout^{18,19} have the disadvantage that they are not capable of multiplex analysis of more than one target in a single reaction chamber.

In alternative detection strategies, MNAzymes can be linked to various target amplification technologies including real-time PCR. To date, no binary or split DNAzymes have been described as being capable of functioning at the elevated temperatures (≥ 50 °C) required for PCR. We demonstrated the use of MNAzyme real-time PCR to quantify a target gene present in genomic DNA and detected as little as 10 copies. The method is highly sensitive and has enhanced specificity compared to other real-time PCR protocols since two partzymes and two PCR primers are required to bind for amplification and detection. A series of generic substrates has been successfully used in multiplex MNAzyme real-time PCR assays that simultaneously quantified five RNA transcripts.²⁰ Furthermore, multiplex MNAzyme real-time PCR was also used to follow an RNA transcript (OZ1 RNA) and a control transcript in an international phase II gene therapy clinical trial,²¹ demonstrating the robustness and utility of the technology in a clinical setting.

MNAzymes are highly versatile enzymes encompassing a range of structures with variant oligonucleotide composition and structure. For example, MNAzymes may incorporate elements such as partzymes with truncated sensor arms and stabilizer arm oligonucleotides, and/or they may require multiple assembly facilitator components for functionality. This further broadens their range of utility to encompass applications such as base-sensitive biosensor elements and computational switches (reviewed in ref 2). Partzymes with truncated sensor arms, together with stabilizer arms, are particularly useful for detection of sequence variations including mutations and single nucleotide polymorphisms (SNPs). In this report, detection of a single base change was dependent upon hybridization at 46 °C between a five-base truncated sensor arm and a target. It is likely that the truncated sensor arm and the adjacent stabilizer arm synergistically cooperated by coaxial helix stacking to complete the complex. The position within the sensor arm and the specific mismatch both influence the degree of discrimination. Many authors have reported that certain mismatches, for example, C-A or G-A, have a greater destabilizing effect on duplexes than others such as G-T. Experiments to date indicate these rules are also useful for predicting mismatches between a target assembly facilitator and a partzyme sensor arm that result in inactive complexes. While the experiments reported in this study used an isothermal, direct detection format, we have since used partzymes with truncated arms for mutation and SNP detection in an MNAzyme real-time PCR (data not shown).

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In another example, MNzyme structures were formed with the use of multiple assembly facilitator component oligonucleotides, all of which were required for enzyme activity. Alternative inactive complexes were formed when partzymes bound to an assembly inhibitor. This produced an MNzyme with a “switch”-like effect such that the absence of an assembly facilitator component, and/or the presence of an assembly inhibitor, prevented formation of an active enzyme and resulted in catalytically inactive or “off” complexes. In contrast, the presence of all abutting assembly facilitator components resulted in catalytically “on” MNzymes. Combining the functions of an MNzyme that is ready to receive input and produce output along with the ability to be turned off and on provides the basis of molecular switches and provides useful components of nucleic acid cascades. These structures can be used for transducing signals, facilitating molecular computation, and/or providing structural elements useful for bioengineering nanoscale “machines”.

Our laboratory has exploited MNzyme structures to develop cascade protocols that may be linked to any isothermal MNzyme assay to increase the sensitivity of target detection and/or amplify the signal. The cascade strategy employed here is controlled by a molecular switch where an initiating MNzyme controls the formation of a second cascading MNzyme. The target-dependent assembly of the initiating MNzyme is required to produce an essential component of the cascading MNzyme, and thus the initiating MNzyme is a molecular switch to turn the cascading MNzyme on. While it was useful to label the two substrates with different fluorophores to provide evidence of both stages of the cascade in this example, labeling both substrates with the same fluorophore would be advantageous in an analytical setting, as this would increase the degree of signal amplification afforded for each target molecule.

This study demonstrates the power of MNzymes, which are inherently more versatile than the antecedent DNAzyme on which they were based. In general, DNAzymes or ribozymes recognize and modify a target substrate; however, the separation of target recognition from substrate modification in the MNzyme design affords increased flexibility and functionality. Further, our study discloses a general method for generating new MNzymes from other nucleic acid enzymes. MNzymes use stable nucleic acid components and this will facilitate simple, fast, and cost-effective methods for the detection of targets.

Conclusion

MNzymes are a powerful class of enzyme that can be activated by the input of any specific synthetic or biological nucleic acid to produce an amplified output, which can be generic in nature. Furthermore, their activity can be switched on or off by the presence or absence of other nucleic acid sequences, and they can be linked in series to produce a molecular cascade or signal transducer. The extraordinary properties of MNzymes confers potential for their integration into diverse devices such as diagnostic biosensors, molecular computers, and/or nanoscale machines.

Experimental Methods

Isothermal, Direct Detection Method. Reactions contained a bulk mix consisting of 0.2 μM each of partzyme A and partzyme B, PCR buffer II (Applied Biosystems), and 25 mM MgCl_2 , (unless stated otherwise). In addition, reactions may also have contained a stabilizer, an assembly facilitator or assembly facilitator component(s), mutated assembly facilitator, or no assembly facilitator (no

AF control reaction). Reactions were initiated by the addition of 0.2 μM substrate. The total volume of each reaction was 25 μL . Reactions were incubated at various, constant temperatures in a SmartCycler System thermocycler (Cepheid), and fluorescence was monitored over time. All samples were run in either duplicate or triplicate. A substrate-only reaction was performed to enable subtraction of the background level of fluorescence from the reactions. Results in all figures are the averages from replicates and were plotted by use of Excel (version 11).

Substrates. MNzyme activity was monitored by cleavage of a dual-labeled nucleic acid substrate composed of DNA (uppercase) and RNA (lowercase) bases. Underlined bases indicate the position of a 6-FAM (F) or Texas Red (TR) moiety on the 5' side of the RNA bases and the Black Hole Quencher moiety (B) on the 3' side of the RNA bases. The Sub2 and Sub6 series of substrates were used with Mz1 MNzymes. Sub2 was either end-labeled (Sub2-FB, AAGGTTTCCTCguCCCTGGGCA) or internally labeled (Sub2i-FB, AAGGTTTCCTCguCCCTGGGCA). Sub6 was end-labeled (Sub6-TRB, ATCACGCCTCguTCCTCCCAG). The substrate used for studies of Mz2 MNzymes was Sub1i-FB, which was internally labeled (ACTCACTATaGGAAAGAGATG).

Partzymes. Partzyme sequences used in these experiments are displayed such that the bases shown underlined form part of the catalytic core of the assembled MNzyme, bases shown in boldface type hybridize with the assembly facilitator, and bases shown in italic type hybridize to the substrate. The presence of “-P” indicates 3' phosphorylation of the partzyme, which prevents extension when partzymes are used in conjunction with PCR.

Assembly Facilitators. The assembly facilitators (AF) AF-RO3 (CAGTGACTTCACATGGGGCAATGGACCAGCACGGGCA-GCAGCTGGC), AF-RO4 (GCCATTGTGCAACACCTGCTG-GATGACCAGC), and AF-RO5 (GAAGGTGTAATCCGTCTC-CACAGACAAGGCCAGGACTCGTTTG) and assembly facilitator component (AFc) AFc1-RO1 (ACTTCCAGGTCGCCCTGTCT-TCCCTGAGC) were homologous to sequence from the human RPLP0 gene (NM_001002). AFc2-RO1 (ATCACGCCTCG) was homologous to the 5' side of Sub6. The assembly facilitator AF-XdT(TGCCCCCTCACCTCACGAAGGTATACAGACAGATGGACATCCAGTTGGTGA) was homologous to the human PLXNB1 gene (NM_002673), and AF-XdC (TGCCCCCTCACCTCACGAAGGCATACAGACAGATGGACATCCAGTTGGTGA) was homologous to the mutant A5359G²² of the human PLXNB1 gene.

Testing Candidate Partzyme Pairs for Cleavage Activity. Reactions were performed in triplicate and were monitored in real time at 9 s intervals for 15 min at 55 °C by the isothermal direct detection method (see above) with the modification of 50 mM MgCl_2 . Every reaction contained Sub2i-FB. Test reactions for Mz1 candidate partzyme pairs (a–g series; see Table S1 in Supporting Information) each contained 0.2 μM AF-RO5 or no assembly facilitator. Test reactions for the 10–23 DNAzyme also contained 0.2 μM Dz1 (TGCCAGGGAGGCTAGCTACAACGAGAG-GAAACCTT).

Cleavage Reactions with Radiolabeled Substrate. Substrate Sub2-FB (1 μM) was 5' end-labeled in 10 μCi [γ -³²P]ATP (Perkin-Elmer), 1 \times PNK buffer, and 1 unit of polynucleotide kinase (New England Biolabs) at 37 °C for 30 min. The reaction contained 1 \times Immobilizer (Bioline), 50 mM MgCl_2 , 5 nM labeled Sub2-FB, and either 100 nM 10–23 deoxyribozyme (Dz1) or 100 nM MNzyme [100 nM each of RO5Ac/2-P (CAAACGAGTCCTGGCCT-TGTCTTACAACGAGAGGAAACCTT-P), RO5Bc/2-P (TGC-CCAGGGAGGCTAGCGTGGAGACGGATTACACCTT-C-P), and AF-RO5] or no enzyme. Reactions were incubated at 53 °C for 30 min. The reactions were stopped by adding formamide loading dye containing 100 mM ethylenediaminetetraacetic acid (EDTA). Upon completion of the reactions, the uncleaved substrate and cleavage

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products of the deoxyribozyme and the MNAzyme were resolved by electrophoresis on a 16% denaturing polyacrylamide gel.

Analysis of MNAzyme Activity. Reactions reported in Figure 3B were monitored at 36 s intervals for 1 h at 40 °C by the isothermal direct detection method (see above) with the modifications of 50 mM MgCl₂ and 50 mM Tris HCl (pH 9.0 at 25 °C). Test reactions also contained Sub1i-FB, RO4Aa/1 (**GCTGGT-CATCCAGCAGCGGTGAAATAGTGAGT**), and RO4Ba/1 (**CATCTCTCTCCGAGCGGTGTTCCGACAATGGC**) in the presence of 0.2 μM AF-RO4 or in the absence of an assembly facilitator (no AF). Control reactions included RO4Aa/1 with 0.2 μM AF-RO4 (no partzyme B, control 1); RO4Ba/1 with 0.2 μM AF-RO4 (no partzyme A, control 2); mutated partzyme A (RO4Aa/1mut **GCTGGT-CATCCAGCAGCGGTGAAATAGTGAGT**) with RO4Ba/1 and 0.2 μM AF-RO4 (control 3); and RO4Aa/1 and RO4Ba/1 with 0.2 μM AF-RO5 (control 4).

Kinetics of MNAzyme Catalysis. Three independent experiments were performed, with each reaction done in triplicate and monitored in real time at 18 s intervals for 15 min at 55 °C. The isothermal direct detection method (see above) was used with the modifications of 50 mM MgCl₂ and various concentrations of Sub2i-FB (either 1000, 750, 500, 250, 200, 150, or 100 nM). MNAzyme test reactions used the Mz1d split (Table S1 in Supporting Information) and contained 1 μM RO5Ad/2-P, 1 nM RO5Bd/2-P, and 1 μM AF-RO5 (equivalent to a maximum concentration of assembled MNAzyme of 1 nM). The 10–23 DNAzyme reaction contained 1 nM Dz1. To enable the conversion of fluorescence data into reaction velocities, a standard curve was generated from a range of concentrations (1000, 500, 250, 125, 62.5, or 31.1 nM) of totally cleaved substrate. The Michaelis–Menten equation was fitted to the data generated from the enzymatic reactions (by use of Prism version 4.0, GraphPad Software Inc.) to derive the enzymatic parameters V_{\max} and K_m . The k_{obs} values were obtained by fitting a hyperbolic function (by use of Prism version 4.0, GraphPad Software Inc.) to the fluorescence versus time data.

Limit of Detection of an Assembly Facilitator. Reactions in Figure 3C were performed in duplicate and were monitored at 72 s intervals for 2 h at 52 °C by the isothermal direct detection method. All reactions contained RO3Ad/2-P (**AGCTGCTGCCCGTGCTG-GTGACAACGAGAGGAAACCTT-P**), RO3Bd/2-P (**TGCCCAGG-GAGGCTAGCTCCATTGCCCATGTGAAGTCA-P**), and Sub2i-FB. Test reactions contained various concentrations of target AF-RO3 (750, 500, 250, 50, 10, or 5 pM) or no assembly facilitator (no AF).

Quantitation of a Nucleic Acid Target via Real-Time PCR. Human genomic DNA extracted from the IM9 cell line (American Type Culture Collection) was used as template for amplification of the K-ras gene (NT_009714.16). Real-time PCR amplification and quantification of the target sequence was performed in a total reaction volume of 25 μL. All reactions were conducted in an Mx3000p system (Stratagene). The cycling parameters were 95 °C for 10 min, followed by 5 cycles of 95 °C

for 30 s and 55 °C for 60 s, and followed by 40 cycles of 95 °C for 30 s and 53 °C for 60 s. The reactions contained 40 nM forward primer (5K-ras TAGTGTATTAACCTTATGTGTGAC) and 200 nM of each reverse primer (3K-ras AATGATTCTGAATTAGCTGTATC), K-rasAd/2-P (TAAACTTGTGGTAGTTGGAGACAACGAGAGGAAACCTT), K-rasBd/2-P (TGCCCAGGGAGGCTAGCTCTGGTGGCGTAGGCAAGAGTGCC), and Sub2-FB, 8 mM MgCl₂, 10 units of RNasin (Promega), 1× Immobuffer (Bioline), 2 units of Immobilase (Bioline), and either genomic DNA template (100 ng, 20 ng, 4 ng, 800 pg, 160 pg, or 32 pg) or no DNA (H₂O).

Discrimination of a Single Base Difference. Reactions in Figure 5A were performed in duplicate and were monitored at 72 s intervals for 2 h at 46 °C by the isothermal direct detection method. All reactions contained XdAd/2-P (**ACTGGATGTCCATCTGTCT-GACAACGAGAGGAAACCTT-P**), XdBd/2-P (**TGCCCAGGGAG-GCTAGCTTATGC-P**), and Sub2i-FB. Reactions also contained 0.2 μM XdSA-P (**CTTCGTGAGGGTGAG-P**) in the presence of either 10 nM AF-XdC or 10 nM AF-XdT or no assembly facilitator. An additional control reaction contained 10 nM AF-XdC but lacked XdSA-P.

Molecular Switch Assay. Reactions in Figure 5B were performed in duplicate and were monitored at 36 s intervals for 1 h at 47 °C by the isothermal direct detection method. All reactions contained RO1Ad/2-P (**GCTCAGGGAAGACAGGGCGACCT-GACAACGAGAGGAAACCTT-P**), RO1Bd/2-P (**TGCCCAGG-GAGGCTAGCTGAAGTCCGAGGCGTGAT**), and Sub2i-FB. In addition, reactions also contained either 0.2 μM AFc1-RO1 and AFc2-RO1, or 0.2 μM AFc1-RO1, or 0.2 μM each of AFc1-RO1 and assembly inhibitor AI (Sub6-TRB), or no assembly facilitator components.

Molecular Cascade Assay. Reactions in Figure 5C were performed in duplicate, and fluorescence of both Texas Red and FAM were monitored at 36 s intervals for 1 h at 48 °C by the isothermal direct detection method. Reactions contained the components of the initiating MNAzyme, RO5Ad/6-P (**CAAACGAGTCTGGCCT-TGTCTACAACGAGAGGCGTGAT-P**), RO5Bd/6-P (**CTGGGAG-GAAGGCTAGCTGTGGAGACGGATTACACCTTC-P**), and Sub2i-FB, and the cascading MNAzyme (RO1Ad/2-P, RO1Bd/2-P, Sub2i-FB, and 0.2 μM AFc1-RO1). In addition, reactions either contained 10 nM target AF-RO5 or lacked target.

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Supporting Information Available: Three tables and three figures, showing sequences and cleavage activity data for the Mz1 and Mz2 candidate partzymes pairs and catalytic activity of Mz1 MNAzyme at various temperatures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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