Catalytic Molecular Beacons

Milan N. Stojanovic,* Paloma de Prada, and Donald W. Landry[a]

We have constructed catalytic molecular beacons from a hammerhead-type deoxyribozyme by a modular design. The deoxyribozyme was engineered to contain a molecular beacon stem – loop module that, when closed, inhibits the deoxyribozyme module and is complementary to a target oligonucleotide. Binding of target oligonucleotides opens the beacon stem – loop and allosterically activates the deoxyribozyme module, which amplifies the recognition event through cleavage of a doubly labeled fluorescent substrate. The customized modular design of catalytic molecular beacons allows for any two single-stranded oligonucleotide sequences to be distinguished in homogenous solution in a single step. Our constructs demonstrate that antisense conformational triggers based on molecular beacons can be used to initiate catalytic events. The selectivity of the system is sufficient for analytical applications and has potential for the construction of deoxyribozyme-based drug delivery tools specifically activated in cells containing somatic mutations.

KEYWORDS:

deoxyribozymes · DNA recognition · fluorescence spectroscopy · molecular beacons · nucleic acids

Introduction

The ongoing elucidation of the molecular basis of disease[1] has increased the demand for methods that discriminate specific nucleic acid sequences.[2, 3] We were interested in nucleic acid discrimination at the resolution of a single base mutation for two applications: (1) the development of simple single-step homogenous analytical methods that could be adapted to high-throughput screening and (2) the creation of novel therapeutic agents specifically activated in cells containing somatic mutations. For both applications, a conformational trigger based on a molecular beacon seemed an excellent starting point.

Traditional molecular beacons[4] are attractive biosensors for the detection of specific nucleic acid sequences in homogenous solution. Molecular beacons are doubly end-labeled oligonucleotides that exist in solution as stable stem – loop structures in which the fluorescence of a reporter dye (**D**) attached to the 5′ end is quenched by a proximate quencher (**Q**) attached to the 3′ end (Figure 1). In the presence of a complementary nucleic acid the stem opens, and this event is signaled by a loss of quenching and an increase in fluorescence. Even a single mismatch can be sufficient to prevent the opening of the stem. However, the signal is limited by the 1:1 stoichiometry of the complex and, thus, the method is usually coupled to polymerase chain reaction (PCR) amplification and real-time PCR detection. An alternative approach that incorporates signal amplification would be to couple the specific oligonucleotide recognition event of a molecular beacon to an enzymatic activity with colorimetric or fluorogenic end points. Thus, the target oligonucleotide would serve as an effector of enzymatic activity. There are previous examples[5] of oligonucleotide-based catalysts allosterically promoted by specific oligonucleotides, but they are not generally applicable and require reselection.

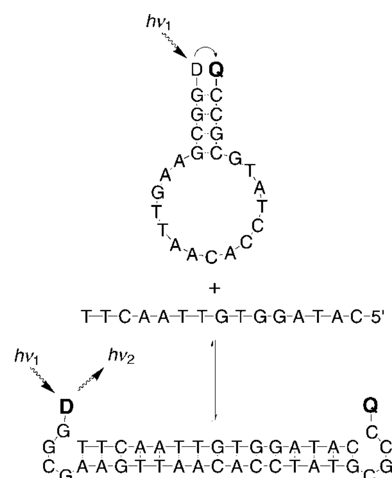

*Figure 1. Molecular beacon signaling the presence of a complementary oligonucleotide by an increase in donor (**D**) fluorescence upon stem opening and separation from the quencher (**Q**).*

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Supporting information (behavior of catalytic molecular beacon (CMB) with fully complementary loop to **1** in the presence of substrate and target oligonucleotides; reaction scheme of CMB with more reactive deoxyribozyme module and modified substrate; spectra of reactions with and without oligonucleotides; fluorescence versus time curves in the presence of target oligonucleotides) for this article is available on the WWW under http://www.chembiochem.com or from the author.

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Based on this analysis, we designed catalytic molecular beacons (CMBs) as modular deoxyribozymes under allosteric control[6] of single-stranded target oligonucleotides. When combined with the appropriately labeled substrate, catalytic molecular beacons can discriminate between target and mutant oligonucleotides that differ in a single base. Furthermore, the method described here is general, that is, any oligonucleotide sequence can be turned into a controlling element by a simple exchange of the recognition element in the beacon module.

Results and Discussion

Design of catalytic molecular beacons

Stoichiometric molecular beacons directly signal the recognition of target oligonucleotides through loss of fluorophore quenching in the open-form hybrid. In contrast, we designed catalytic molecular beacons, such that the recognition of the target oligonucleotide activates a hammerhead-type deoxyribozyme[7] (Figure 2, plain font) possessing RNase activity. The Mg^{2+}-dependent deoxyribozyme has a reported turnover of $0.04\,min^{-1}$ for hydrolysis of a 15-mer oligonucleotide substrate **S** containing a single ribonucleotide embedded in a deoxynucleotide framework. We attached to the 5′ terminus of the deoxyribozyme a beacon module configured to complement the target oligonucleotide **1** (d(CATAGGTGTTAACTT)), such that the stem–loop structure of the molecular beacon formed an independent domain within the larger oligonucleotide.[8] We extended the beacon stem to overlap with the 5′ substrate recognition domain and to completely inhibit catalytic cleavage in the absence of the target oligonucleotide. We expected that, by analogy to stoichiometric molecular beacons, the addition of target oligonucleotide would open the stem of the beacon module and thereby permit substrate binding and cleavage. The overlap between the substrate recognition region and beacon stem was carefully optimized so as not to suppress turnover in the activated form. The optimal overlap length for recognition of the pentadecadeoxyoligonucleotide with ca. 40% GC content was five base pairs.

The ability of a sensor to function in homogenous solution hinges on the reporter function. Despite significant advances in the design of ribozymes, until recently no ribozyme-based reaction had been coupled to an optical read-out system in homogenous solution. To achieve our purpose, we devised a simple method based on a transformation of fluorogenic substrate.[9] Recently, this technique has been used for kinetic characterization of a hammerhead ribozyme,[10] and we have demonstrated[11] that general fluorogenic homogenous assays based on deoxyribozyme catalysis are possible, including the recognition of a single-base difference in a special case where the oligonucleotide was complementary to the part of the parent deoxyribozyme. Also, a similar method has been recently combined with PCR to yield DzyNA-PCR, wherein the amplicons that contain a deoxyribozyme cleaved a reporter substrate in clinically relevant samples.[12]

We placed a fluorescein donor (**F**) at the 5′ terminus of **S** and its fluorescence emission was partially quenched by the tetramethylrhodamine acceptor (**R**) positioned at the 3′ terminus. Cleavage of doubly end-labeled substrate to products $\mathbf{P_1R}$ and $\mathbf{P_2F}$ (Figure 2) resulted in a tenfold increase in fluorescein emission at 520 nm (excitation at 480 nm).

Catalytic molecular beacons are selectively activated by a target oligonucleotide

We tested the ability of the catalytic molecular beacon to distinguish 15-mer oligonucleotides: **1** (d(CATAGGTGTTAACTT)), **2** (d(CATAGGTCTTAACTT)) with a G8 to C8 substitution versus **1**, and **3** (d(CATAGGTGTTTACTT)) with an A11 to T11 substitution versus **1**. The deoxyribozyme with the beacon module fully complementary to **1** was triggered at room temperature preferentially by **1** over **2**, but showed no selectivity over **3** (see Supporting Information). However, we noted that double mutants were much less effective in triggering the enzymatic activity. Thus, in order to optimize the differentiation of **1** from **2** and **3** at room temperature we introduced a single-base mismatch in the beacon module (A_{19} in Figure 2) as an "auxiliary mutation" to obtain **CMB-1**. **CMB-1** possesses one mismatch with respect to **1** and two mismatches with respect to **2** and **3**. When the deoxyribozyme **CMB-1** was mixed with substrate in the presence of oligonucleotide **1**, or the "mutants" **2** or **3**, it was clearly capable of distinguishing them (Figure 3 A). Initial experiments were performed with an

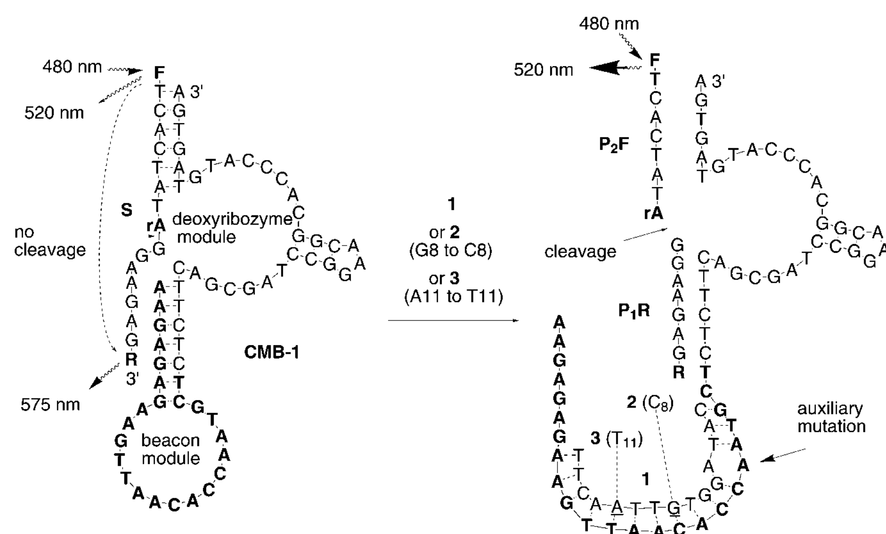

*Figure 2. Catalytic molecular beacon **CMB-1**, substrate **S** with embedded ribonucleotide (**rA**) (shown in the nonproductive complex with deoxyribozyme), target oligonucleotide **1** (shown in the complex with activated **CMB-1**) and products $\mathbf{P_1R}$ (**R** = tetramethylrhodamine) and $\mathbf{P_2F}$ (**F** = fluorescein). **CMB-1**: Plain font = parent deoxyribozyme; bold font = beacon module. Upon binding of **1** cleavage occurs and fluorescein emission increases. Compared to **1**, oligonucleotides **2** and **3** have G8 to C8 and A11 to T11 mutations (see underlined bases).*

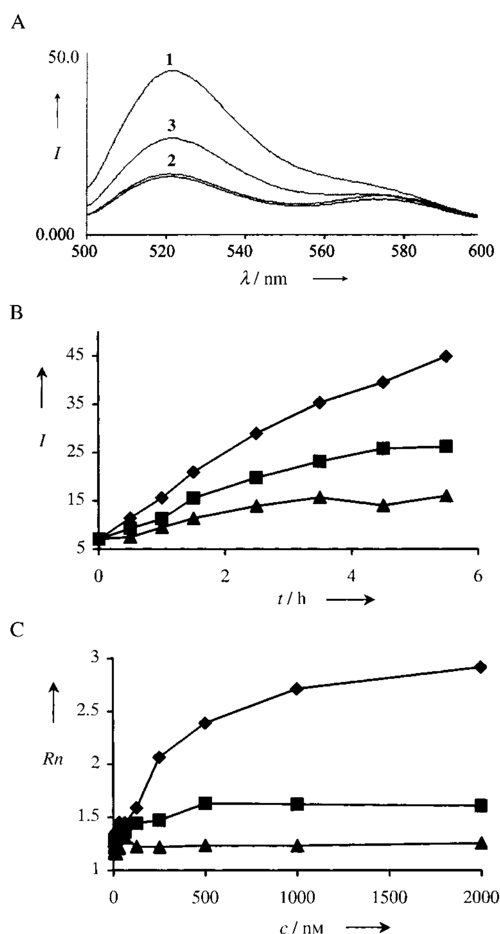

*Figure 3. A: Fluorescence spectra of reactions at t = 5.5 h, in the presence of (from top to bottom): **1**, **3**, **2** and no oligonucleotide; conditions: c(Mg^{2+}) = 10 mM, c(**CMB-1**) = 1 μM, c(**S**) = 10 μM, c(**1**, **2** or **3**) = 500 nM. B: Fluorescence intensity versus time in the presence of target nucleotides **1** (◆), **3** (▲), **2** (■); conditions as described for Figure A. C: Rn value (E_{520}/E_{575}) versus concentration of oligonucleotides **1** (◆), **3** (▲), or **2** (■) at t = 18 h; conditions: c(Mg^{2+}) = 10 mM, c(**CMB-1**) = 50 nM, c(**S**) = 2 μM.*

excess of **CMB-1** (1 μM) to render the target oligonucleotide (0.5 μM) the limiting reagent. Substrate concentrations corresponded to the K_m value of the parent deoxyribozyme (ca. 10 μM). Figure 3 B shows the time course of fluorescein emission. Oligonucleotide **1** was distinguished easily from **2** and **3** within 30 min after ca. 1.4 turnovers per molecule of activating oligonucleotide **1**. Less than 0.2 turnovers were observed with **2** and less than 0.7 turnovers per molecule of **3**. We were able to achieve clear fluorescent end points even after 5.5 h at room temperature, with ca. 12 turnovers per molecule of **1** (ca. 3 turnovers per molecule of **2**, ca. 6 turnovers per molecule of **3**). These data demonstrate the capability of the molecular beacon stem – loop to confer selective allosteric activation by **1** over **2** or **3**. As expected, mutations that interrupt A-T pairing (as in **3**) are more effective in activation than mutations that affect the G-C pair (as in **2**).

To characterize this system, we studied the sensitivity of the catalytic molecular beacon (at c = 50 nM) in the presence of oligonucleotides at various dilutions and time points. At lower deoxyribozyme concentration, the cleavage reaction without activating oligonucleotides and with **2** was negligible, and prolonged incubation times could be used to maximize sensitivity. For this experiment we define the *Rn* value as a ratio of the emission at 520 nm from fluorescein and emission at 575 nm from rhodamine after excitation at 480 nm. At low turnover, when both rhodamine and fluorescein emission maxima are clearly separated, this representation has an advantage because rhodamine emission serves as an internal standard, thus minimizing experimental variations. Figure 3 C shows the influence of oligonucleotide concentration on the reaction progress, expressed as the *Rn* value at t = 18 h. **CMB-1** is promoted by **1** at 8 nM as efficiently as by **2** at 1 μM, and we were able to detect **1** at a concentration of less than 8 nM.

Catalytic molecular beacons are fully modular

We expected that catalytic molecular beacons would share generality with their noncatalytic counterparts and that the beacon module could be substituted to specifically recognize other oligonucleotides. To test this possibility, we constructed deoxyribozyme **CMB-2** with a beacon module that would signal the presence of oligonucleotide **2**, but not **1** (Figure 4). The

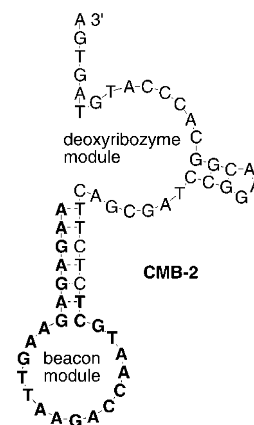

*Figure 4. Catalytic molecular beacon **CMB-2**, shown in the nonproductive conformation. **CMB-2** differs from **CMB-1** in a substitution of C15 to G15 in the beacon module (underlined base).*

deoxyribozyme **CMB-2** contains the same deoxyribozyme module, but a stem – loop module complementary to **2** (except for the same A_{19} auxiliary mutation). Instead of oligonucleotide **3**, we tested oligonucleotide **4** (d(CATAGGTCTTTACTT)) with an A11 to T11 substitution as compared to **2**. **CMB-2** possesses two mismatches with respect to **4** and should behave as **CMB-1** did in the presence of **3**. All these prediction were born out: **CMB-1** and **CMB-2** differed in only one position, C_{15} versus G_{15}, but their responses to **1** and **2** were exactly the opposite. Deoxyribozyme **CMB-2** catalyzed the fluorogenic reaction in the presence of **2** and showed little activity in the presence of **1** (Figure 5 A). Also, **CMB-2** behaved in the presence of **4** as did **CMB-1** in the presence of **3**, further demonstrating the modular behavior of this system. **CMB-2** was not active in the presence of triply

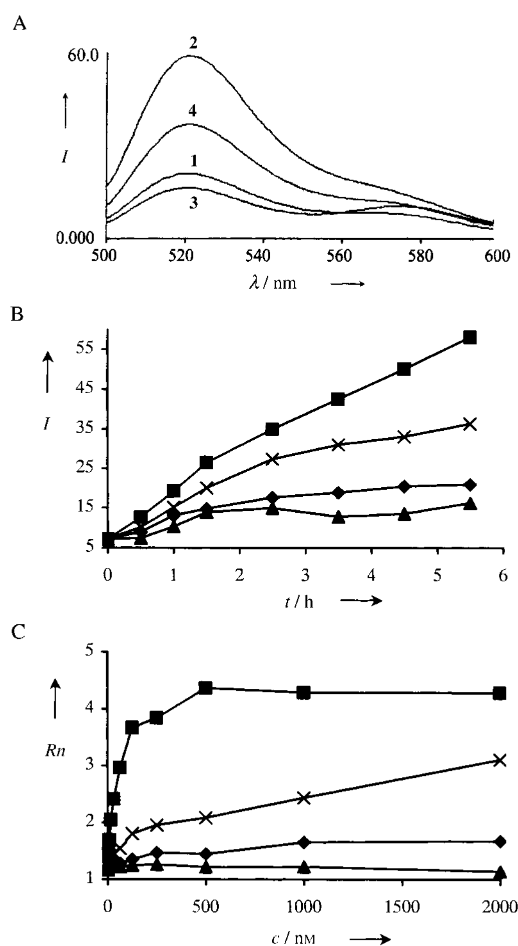

Figure 5. A: Fluorescence spectra at t = 5.5 h in the presence of (from top to bottom) ***2***, ***4***, ***1***, *and* ***3****; conditions: c(Mg^{2+}) = 10 mM, c(**CMB-2**) = 1 μM, c(**S**) = 10 μM, c(**1**, **2**, **3** or **4**) = 500 nM. B: Fluorescence intensity versus time in the presence of target oligonucleotides **1** (◆), **3** (▲), **2** (■), and **4** (×), conditions as described for Figure A. C: Concentration of oligonucleotides **1** (◆), **3** (▲), **2** (■), and **4** (×) versus Rn value (E_{520}/E_{575}) at t = 20 h; conditions: c(Mg^{2+}) = 10 mM, c(**CMB-2**) = 100 nM, c(**S**) = 2 μM.*

mismatched **3**. Oligonucleotide **2** can be detected by **CMB-2** within thirty minutes (Figure 5B) after ca. 1.7 turnovers per molecule of **2** (after < 0.6 turnovers per molecule of **1**). **CMB-2** is promoted by **2** at 8 nM as effectively as by **1** at 2 μM (Figure 5C), and we were able to detect **2** at a concentration of less than 2 nM.

Conclusions

We have successfully demonstrated that molecular beacon loops can be used to selectively trigger a secondary catalytic event, in this case a deoxyribozyme reaction. Furthermore, through coupling of this reaction to fluorescence reporting, we generated "catalytic molecular beacons" that can report the presence of mutations in single-stranded nucleic acids. In this design, the molecular beacon module interacts with a target oligonucleotide and a deoxyribozyme module amplifies this signal. The beacon can be configured through auxiliary mutations to operate at a selectivity threshold of a single point mutation. We note that the increase in fluorescence per substrate turnover is severalfold smaller than the increase following the stem – loop opening in the stoichiometric beacons, and this is currently the obstacle for immediate analytical applications of the catalytic molecular beacons. Despite high background fluorescence of the substrate and the low turnover number of this deoxyribozyme, the demonstration that a molecular beacon stem – loop can be used to activate deoxyribozymes is important, as it is general and can be expanded to other, more active deoxyribozymes. For example, we have used similar beacon modules to control a more active deoxyribozyme[14] with various substrates and have decreased the time of detection and background fluorescence (see Supporting Information). Any mRNA that is currently being tested in antisense therapy can be now considered for an activator of ribozyme activity by simply combining the appropriate stem – loop and enzyme modules, and we are currently exploring potential antisense therapeutic applications of deoxyribozymes activated by specific sequences.

Experimental Section

Materials: All oligonucleotides were custom-made by Integrated DNA Technologies, Inc. (Coralville, IA), and purified by HPLC or PAGE electrophoresis, except 15-mers **1**, **2**, **3** and **4** that were used desalted. Samples were dissolved in RNase- and DNase-free water, separated in aliquots, and frozen at −20 °C until needed. All experiments were performed in autoclaved buffer (50 mM HEPES, 1 M NaCl, pH 7.5) at room temperature. $MgCl_2$ was obtained from Sigma – Aldrich Co. (St. Louis, MO) and used as 200 mM autoclaved stock solution in water.

Spectroscopic measurements: All fluorescence spectra were obtained on an F-2000 Fluorescence Spectrophotometer (Hitachi Instruments Inc., San Jose, CA) equipped with a xenon lamp (Hamamatsu). Experiments were performed at the excitation wavelength of 480 nm and emission scans at 510 – 590 nm.

Catalytic molecular beacons, target oligonucleotides, and labeled substrate were diluted to the desired concentrations in HEPES buffer and mixed in that order. Reactions were initiated after 10 min by the addition of Mg^{2+}. The total reaction volume was 20 μL. Aliquots (2.5 μL) were diluted to 0.5 mL with HEPES buffer and transferred into a quartz microcuvette. Peak selection at the two maxima (519 – 522 nm, or 570 – 578 nm) was performed automatically, and these values were used in calculations of the *Rn* value ($Rn = E_{520}/E_{575}$).

Determination of substrate turnover: The fluorescence emission of the equimolar mixture of two products $\mathbf{P_1R}$ and $\mathbf{P_2F}$ (1.5 μM each) in the buffer was compared to the fluorescence emission of the substrate **S** (1.5 μM) and the estimate of tenfold increase in fluorescence intensity upon substrate cleavage was obtained. The following formula[13] was used to estimate the product formed at time *t* ($c(\mathrm{P})^t$ in μM):

$$c(\mathrm{P})^t = \frac{E^t_{520} - E^0_{520}}{9E^0_{520}}\, c(\mathrm{S})^0;$$

where E^0_{520} is the fluorescence emission at $t = 0$, $c(\mathrm{S})^0$ is the substrate concentration at $t = 0$ in μM, E^t_{520} is the fluorescence emission at time *t*.

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[14] Note added in proof: After our initial submission a more active deoxyribozyme was reported (k_{cat} up to 1 min^{-1}; J. Li, Y. Lu, *J. Am. Chem. Soc.* **2000**, *122*, 10466 – 10467). We adapted a truncated version of this deoxyribozyme to serve as a catalytic molecular beacon and demonstrated its function on **S** and on a substrate containing a ribonucleotide in the stem – loop structure to minimize background fluorescence (see Supporting Information). This system is under intensive investigation, including partial randomization and reselection in order to enhance the turnover of modified substrate.

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