Regulating Enzyme Activities in a Multiple-Enzyme Complex

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DNAzymes have been isolated from synthetic DNA libraries by in vitro selection.[1,2] To regulate the enzyme activities, allosteric DNAzymes have been rationally designed,[3] in which the enzyme activities are modulated by small molecules through ligand–aptamer association. In spite of success of this approach, it has limitations. First, structural information is important for successful design of allosteric DNAzymes, but our knowledge of aptamer and DNAzyme structures is limited. Second, it would be difficult to expand this strategy to specifically control each enzyme if a large number of similar enzymes exist. Herein, we report a general strategy that overcomes these limitations.

A strand-displacement mechanism has been developed for DNA nanomachines.[4] With proper designs, multiple steps of strand displacement have been used for complicated motions.[5] It has also been used to regulate DNA aptamer-binding activity.[6] The key to this mechanism is to remove a partially base-paired DNA strand from a complex by addition of a removal strand; this allows the first strand to become fully base-paired. The number of paired DNA bases increases, and the free energy of the system decreases. This process does not require global denaturation and is sequence-dependent. It only affects strands with specific sequences. All other DNA structures in the same solution remain unchanged. In this paper, we have applied this strategy to regulate the enzyme activities of 10–23 RNA-cleaving DNAzymes.[1]

Multiple-enzyme complexes were modeled with a single DNA strand (E) that contained two 10–23 DNAzymes (E_a and E_b) that were joined by an interenzyme single-stranded linker (Figure 1). A 10–23 DNAzyme has two diverse RNA-recognition arms and a conserved catalytic core. The recognition arms bind to an RNA substrate (S) through Watson–Crick base pairing and determine the enzyme specificity. An *i*nhibitor strand (I) has been designed for each enzyme. Strand I can base-pair with one recognition arm of a DNAzyme and a small part of the catalytic core. Strands I and E form a single duplex domain instead of a bulged duplex, as between strands E and S. Thus, to any DNAzyme, its inhibitor has higher affinity than its substrate. Note that each inhibitor is specific, because inhibitor strands have only negligible affinity to the catalytic cores themselves. Upon binding to strand I, a DNAzyme can not effectively bind to its RNA substrate and loses its RNA-cleaving ability. The enzyme activity can be restored by removal of strand I through strand displacement. Besides the enzyme-binding domain, each inhibitor has a ten-base-long tail, which remains unpaired in the E–I complex. We used a remover

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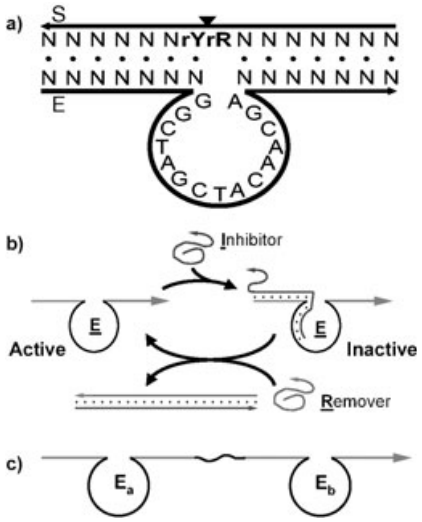

Figure 1. Sequence-dependent regulation of enzyme activities. a) Structure of a 10–23 DNA enzyme (DNAzyme, the bottom strand, E) and its RNA substrate (the top strand, S). The arrow head indicates the cleavage site. The sequence of the conserved catalytic core is shown. Y: pyrimidine, R: purine. b) Reversibly regulating a 10–23 DNAzyme. Addition of the inhibitor strand (I) prevents an enzyme from binding to and cleaving its substrate; subsequent addition of the remover strand (R) displaces strand I and reactivate the enzyme. Strands I and R are fully complementary to each other and form a long duplex. c) Two DNAzymes (E_a and E_b) are joined by a single-stranded linker. The two enzymes differ by binding arms and substrate specificity.

strand (R) to remove I from the E–I complex. Strands R and I have the same length and are fully complementary to each other. These two strands can form a fully base-paired duplex. Strand R base-pairs with strand I at the single-stranded tail first and then gradually pulls strand I out from the E–I complex by branch migration. Upon removal of strand I, the enzyme activity is restored. Each enzyme can be isothermally switched on and off. This strategy has been used to regulate a single DNAzyme activity. Though it is straightforward to extend this strategy to the regulation of multiple DNAzymes, there is no experimental demonstration. We tested this idea in a model system consisting of two DNAzymes. E_a and E_b have different recognition arms and act on different substrates. An inhibitor strand will specifically inhibit one enzyme without affecting the other enzyme. The same is true of the remover strands. A remover strand specifically removes only one inhibitor strand from the E–I complex without affecting the other inhibitor strand.

The inhibition and restoration of each enzyme in the two-enzyme complex was initially demonstrated separately by PAGE. Figure 2a shows that strand I_a binds to strand E to form a stable E–I_a complex, which appeared as a sharp, single band with expected mobility on the gel. Addition of strand R_a frees strand E, and an I_a–R_a duplex forms. Corresponding to the association or removal of strand I_a, the enzyme activity of E_a is inhibited or restored, respectively. The same phenomenon happens to E_b (Figure 2b). These experimental results clearly show that each DNAzyme can be isothermally switched on and off.

The regulation strategy was further demonstrated by sequentially switching the two enzymes off and on (Figure 2c). We started the experiment with both E_a and E_b being active,

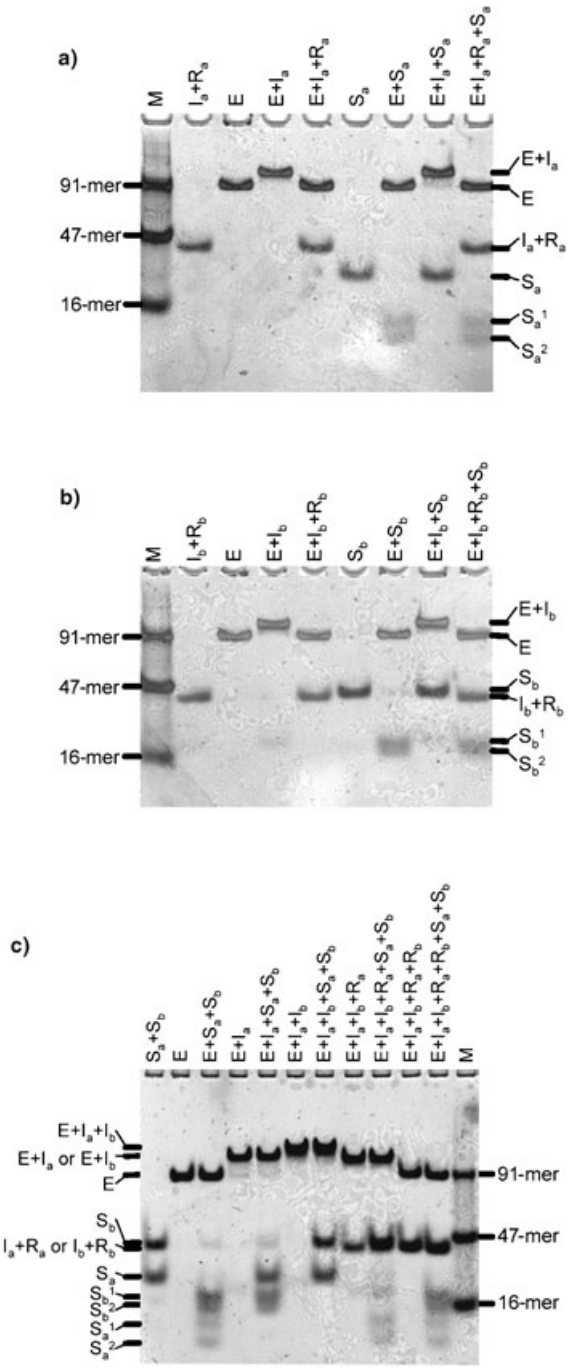

Figure 2. Native gel-electrophoresis analysis of the enzyme activities. In a) and b), only one DNAzyme was switched on and off. In c), the two enzymes were switched off and then on sequentially. After addition of each strand, the solution mixtures were incubated for 30 min before adding the next strand. Following addition of the substrate, the reaction mixtures were incubated for two hours.

then added inhibitors to suppress enzyme activity in a stepwise fashion, and finally used removal strands to remove each inhibitor and restore each enzyme. The sequence is: 1) E_a on, E_b on; 2) E_a off, E_b on; 3) E_a off, E_b off; 4) E_a on, E_b off; and 5) E_a on, E_b on. At each step, two aliquots of the solution were withdrawn. One aliquot was incubated with both substrates (S_a and S_b) and then was analyzed by native PAGE along with the

other aliquot. Experimental results show that, at each step, the desired E–I and/or R–I complexes are formed as expected, and that the enzyme activity is switched on and off as directed. Addition of strand I_a turns off E_a, but E_b is still active. Consequently, S_b is cut and S_a remains intact. Further addition of strand I_b turns off both E_a and E_b, and both S_a and S_b remain intact. Addition of strand R_a removes I_a and frees E_a, resulting in cleavage of S_a. At this stage, I_b still associates with E_b, so S_b remains primarily intact. Note a small amount of S_b fragments is visible, which is probably due to some inhibition leakage. When R_b is further added, both inhibitors are removed, and both enzymes become active; this leads to the cutting of both substrate strands. The sequential switching on and off of these two enzymes unambiguously proves the regulation strategy.

The final proof of this strategy is a time-course experiment of the codigestion of the two substrates by the enzyme complex (Figure 3). Both substrates were incubated with the

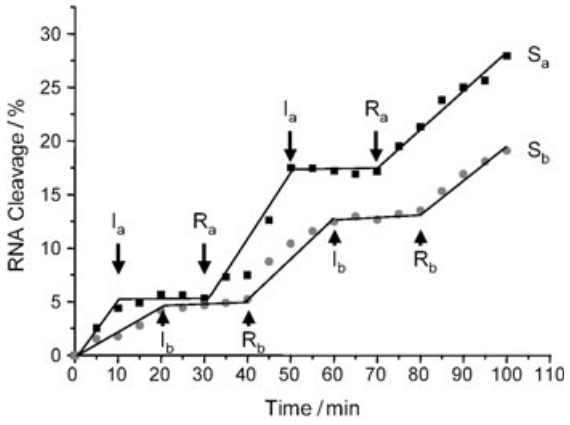

Figure 3. Time course of codigestion of the two substrates (S_a and S_b). Arrows indicate the time points at which inhibitor (I) or remover (R) strands were added. To ensure that enzyme activity was switched on or off, inhibitor or remover strands were used at five times the enzyme concentration. For the purpose of quantification, the substrate molecules were labeled with radioactive isotope ^{32}P and the digestion data were quantified from a PhosphoImager (Packard Instruments). For clear view, the experimental data points were roughly fitted to lines with the expectation of linear kinetics.

enzyme complex in the same solution. Along the reaction course, we sequentially switched off and on each enzyme twice, and monitored the cleavage process of the two substrates. It is clear that that each enzyme is switched on and off independently and reversibly.

A related strategy has been reported to regulate DNAzyme activities.[2e] There, inhibitory strands are covalently linked with DNAzymes or ribozymes. Those enzymes are inactive on their own. Disruption of the inhibition effect by addition of removal strands can switch on the enzymes. However, those studies have only shown that inactive enzymes could be switched on. They have not explored switching enzymes off and on reversibly, or explored the situation when two or more enzymes are physically associated together, though nothing in principle precludes it.

In conclusion, we have applied a strand-displacement strategy to specifically, isothermally regulate individual enzymes in enzyme complexes. The same strategy would be expected to be applicable to other nucleic acid enzymes and aptamers. We consider this work as an initial step to develop highly regulated enzyme complexes to mimic cellular enzyme assemblies. If organized in a large assembly, nucleic acid enzymes/aptamers can, we believe, play more sophisticated roles than individual nucleic acid enzymes/aptamers currently do. This study is important for the following reasons. 1) It shows that nucleic acid enzymes can be isothermally and individually regulated as modern protein enzymes in cells; this is consistent with the hypothesis that nucleic acids played important roles in primitive lives.[7] 2) This study adds new components to biobricks, which are essential for the reconstitution or modification of cell functions. 3) This study paves the way to developing efficient catalysts for technological applications. 4) This study may also be useful for bioanalysis, for example, detection of viral DNA.

Experimental Section

Oligonucleotides. Strand E: 5′-GGA CAG AGG CTA GCT ACA ACG AAG-TGG TAA GCG ATG GGC TAG CTA CAA CGA CCC TTG AGC AGT CAG GCT-AGC TAC AAC GAG ATA GGT-3′; strand S_a: 5′-TTT TTA CCA CTrA rUCT-GTC CTT TTT-3′; strand S_b: 5′-TTT TTT TTT TAC CTA TCrA rUGA CTG-CTT TTT TTT TTT-3′; strand I_a: 5′-TAG CCT CTG TCC GTA TGC GTG A-3′; strand I_b: 5′-TAG CCT GAC TGC CAA ACT GTG C-3′; strand R_a: 5′-TCA-CGC ATA CGG ACA GAG GCT A-3′; strand R_b: 5′-GCA CAG TTT GGC-AGT CAG GCT A-3′. Note that strands S_a and S_b contain different numbers of Ts at both ends; this makes it easier to distinguish different DNA molecules in the gel. Strand E contains E_a and E_b through a linker. The length of the linker is arbitrarily chosen without optimization. The binding sites of the two enzymes are independent from each other. All oligomers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and purified with 10–20% denaturing PAGE.

Radioactive isotope labeling. A solution (20 μL) of primer strand (2 pmol), [γ-^{32}P]ATP (10 mCi mL^{-1}, 1 μL, 2.2 μM), polynucleotide T4 kinase (3 units, New England Biolabs, Inc.) in a kination buffer (66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.6), was incubated for 2 h at 37 °C. The reaction was stopped by heating the reaction mixture at 90 °C for 5 min. Then the labeled DNA strand was purified by 20% denaturing PAGE.

Enzymatic reactions. Reactions were carried out at 22 °C for 2 h in TAE-Mg^{2+} buffer (40 mM Tris buffer (pH 8.0), 20 mM acetic acid, 2 mM EDTA, and 12.5 mM $(CH_3COOH)_2Mg$). The substrate concentration was 10 μM, and the concentration for each of the other strands was 0.5 μM.

Time course of enzymatic digestion. Reactions were carried out at 22 °C for 2 h in TAE-Mg^{2+} buffer, with enzyme (0.1 μM), RNA substrate (1 μM each), and 5′-^{32}P-labeled substrates (0.1 μM each). Inhibitor and remover strands in fivefold excess over enzyme concentration were added to the mixture at the indicated time points. Aliquots (3 μL) were removed from the reaction mixture every 5 min and quenched with EDTA in formamide (7 μL, 40 mM). Radiolabeled substrate and products were separated by denaturing PAGE and quantified by using OptiQuant (Packard Instruments, Meriden, CT, USA).

Denaturing polyacrylamide gel electrophoresis. Gels contained 20% polyacrylamide (acrylamide/bisacrylamide 19:1) and urea (8.3 M). They were run at 22 °C. The running buffer was Tris-borate–

EDTA (TBE), which consisted of Tris buffer (89 mM, pH 8.0), boric acid (89 mM), and EDTA (2 mM). Gels were run on a B-VE10-1 electrophoresis unit (FisherBiotech) at 300 V (constant voltage).

Native polyacrylamide gel electrophoresis. Gels contained 12% polyacrylamide (acrylamide/bisacrylamide 19:1) and were run on a FB-VE10-1 electrophoresis unit (FisherBiotech) at 22 °C and 100 V (constant voltage). The running buffer was TAE-Mg^{2+} buffer. After electrophoresis, the gels were stained with Stains-all dye (Sigma) and scanned.

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