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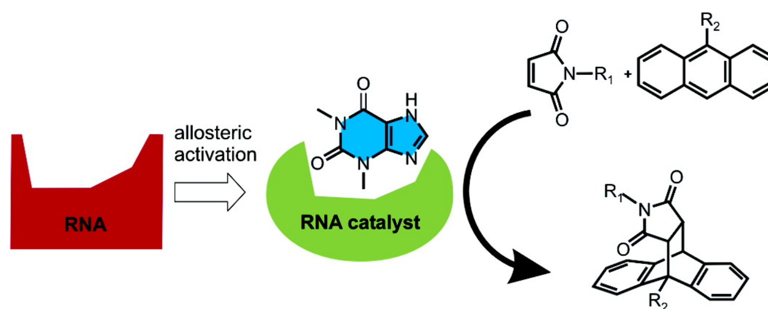

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Allosterically Activated Diels−Alder Catalysis by a Ribozyme

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Biocatalysis is a ubiquitous feature of life, predominantly regulated by allosteric principles. Binding of an effector molecule to a distant site induces a structural change which is communicated to the active center, thereby influencing enzymatic activity. Allosteric activation and inhibition have been successfully applied to develop regulated ribozymes.[1−4] A recent study suggests that this principle might be operative also in nature.[5] All published examples, however, utilize RNA-cleaving or -ligating ribozymes, making a generalization of the structural and mechanistic principles involved difficult. In this communication, we describe the first example in which a ribozyme-catalyzed reaction between two small organic substrates is allosterically regulated by a small organic effector molecule.

Our group has previously described artificial ribozymes that catalyze the formation of carbon−carbon bonds between anthracene and maleimide derivatives by Diels−Alder reaction.[6,7] These ribozymes are active both as self-modifiers (anthracene covalently tethered to the catalyst and maleimide derivatives free in solution) and as true catalysts displaying multiple turnover (both substrates free in solution).[8] For the most-characterized Diels−Alderase ribozyme **1**, the catalytic center is formed by the nucleotides of the asymmetric internal bubble and the nucleotides of the 5′-GGAG-end (Figure 1).[9,10] As stem III was found to be completely variable in sequence and length,[9] it was chosen as the attachment site for a previously described theophylline-binding RNA aptamer.[11] These two functional modules have now been combined in a way that binding of theophylline to the RNA activates the Diels−Alderase.

To identify communication modules optimally suited for the present combination of ribozyme and aptamer,[12] two stretches of five random nucleotides each were inserted in a combinatorial way (**2**).

The resulting library contained 4^{10} ($\sim 10^6$) different potential communication modules between the ribozyme and theophylline aptamer. The 3′-end of the constructs was extended to generate a primer binding site for enzymatic amplification. The previously established *in vitro* selection procedure for Diels−Alderases, which involved anthracene covalently tethered to the RNA, was adapted to allosteric selection in analogy to schemes developed by the Breaker lab.[13]

The pool of DNA templates was transcribed by T7 RNA polymerase in the presence of anthracene−poly(ethylene glycol)−guanosine initiator nucleotides,[6,14] thereby generating library **2** of 10^6 different RNA-tether-anthracene conjugates. This pool was then iteratively deconvoluted over seven rounds of selection and amplification implementing two different kinds of counter-selection to remove the constitutively active ribozymes (i.e., those that are active both in the presence and in the absence of theophylline). During counter-selections in rounds 1−3, the anthracene-initiated pool RNA was reacted with biotin maleimide in the absence of theophylline, and reacted RNA molecules were removed from the pool by immobilization on streptavidin agarose. In subsequent rounds, the counter-selection step was based on a different strategy

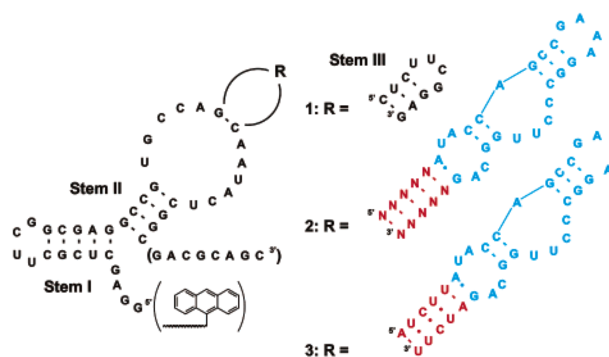

Figure 1. Unregulated and regulated Diels−Alder ribozymes. (**1**) Parent ribozyme;[6] (**2**) ribozyme and theophylline aptamer[11] joined by combinatorial library of potential communication modules; (**3**) selected theophylline-dependent Diels−Alder ribozyme. Black: Diels−Alder ribozyme. Red: communication module. Blue: Theophylline aptamer. Anthracene attached to the 5′-end and a primer binding site attached to the 3′-end are in brackets.

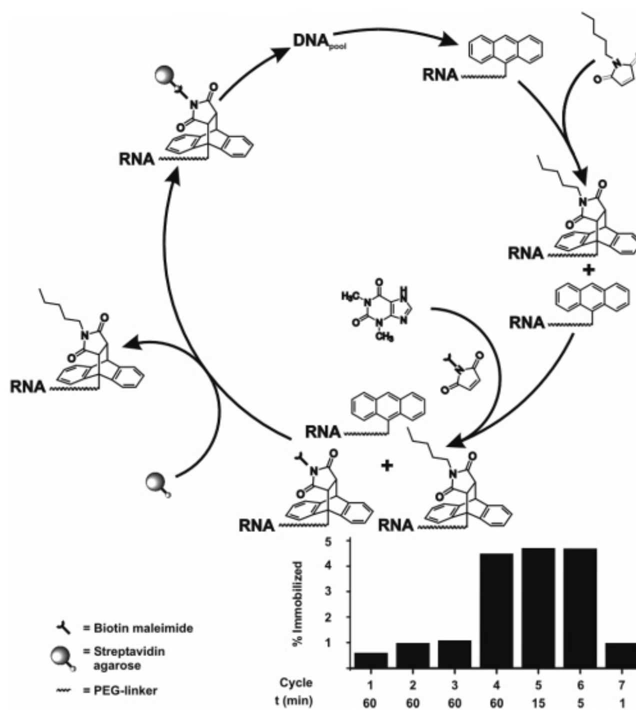

Figure 2. Selection scheme and progress. For details, see online Supporting Information.

(Figure 2). An unselectable substrate, *N*-pentyl maleimide (NPM), which lacks the biotin residue, was reacted with the RNA pool, thereby quenching rather than physically removing the constitutively active molecules.[15]

Excess NPM was then removed by ether extraction. Following this counter-selection, theophylline and biotin maleimide were added

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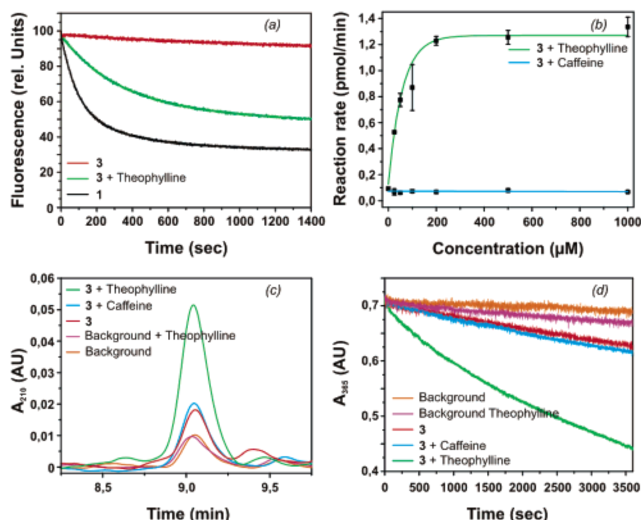

Figure 3. Diels−Alder catalysis by RNA **3** in the absence and presence of 100 μM effector (unless otherwise stated). (a) Fluorescence−time curve for the single-turnover reaction of RNA-tethered anthracene with biotin maleimide with and without theophylline. Parent sequence **1** is included as reference. (b) Dependence of the reaction rate on the concentration of theophylline and caffeine (single turnover). (c) HPLC analysis of multiple-turnover reactions after 1 h reaction time. (d) Absorbance−time curve for the multiple-turnover reaction catalyzed by **3**.

to the aqueous phase. The active RNA molecules were isolated using streptavidin agarose. After a washing step, these selected species were reverse transcribed into cDNA, PCR amplified, and processed to the next round of selection after renewed transcription into RNA.

In round 4, the percentage of immobilized RNA increased by a factor of 4 (Figure 2). Consequently, in subsequent rounds, the selection stringency was gradually increased by a combination of factors, including reduction of reaction time and theophylline concentration. After round 7, the pool was cloned, and individual clones were screened for activity under single-turnover conditions. Clone **3** RNA, showing the strongest activation by theophylline, contains a communication module with two palindromic strands of five nucleotides (Figure 1).

For comparison with the parent Diels−Alderase sequence **1**, the 3′-primer binding site was removed. Single-turnover kinetics (Figure 3a) in the absence and presence of theophylline indicate a ∼20-fold activation of catalysis by theophylline. However, **3** in the presence of theophylline is 3.5-fold slower than the parent sequence **1** (black curve). The single-turnover reaction rates are a function of the theophylline concentration (Figure 3b), with the maximum catalytic rate observed above 100 μM and a half value around 50 μM. The structurally related compound caffeine does not activate the RNA at concentrations up to 1 mM.

Allosteric activation was also investigated in a genuine catalytic system involving free anthracene and maleimide derivatives under multiple-turnover conditions,[7,8] which include concentrations of ribozyme and reactants considerably higher than that in the single-turnover assays (see Supporting Information). HPLC analysis was used to identify the Diels−Alder product by comparison with an authentic sample (Figure 3c). To investigate reaction kinetics in detail, the disappearance of the typical UV absorbance band of anthracene at 365 nm was monitored over 1 h. A 5-fold activation by theophylline could be estimated from the UV traces in Figure 3d (green vs red curve). The consumption of anthracene reaches two turnovers within 1 h. Interestingly and despite the two different counter-selection strategies applied, the reaction rate for **3** in the absence of theophylline is still 3 times higher than the background (no RNA present, red curve vs orange and purple curves). This non-negligible reactivity in the absence of the effector seems to be related to the exceptionally stable tertiary structure of the Diels−Alderase ribozyme.[10] Finally, no significant activation by caffeine was detected (Figure 3d, blue vs red curve).

In conclusion, this is, to our knowledge, the first allosterically regulated ribozyme acting on two small (non-RNA) substrates. Furthermore, this is the first report of allosteric regulation of a catalytic Diels−Alder reaction. The chromophoric and fluorophoric properties of anthracene render this system attractive for the development of assays for a variety of analytes that could be used as effectors, and the observed multiple turnover is equivalent to signal amplification. The primary structures of all three principal components of this allosteric ribozyme, namely, catalytic domain, communication module, and allosteric site, have been isolated from synthetic combinatorial libraries[2] and are not found in nature. While allostericity is a common phenomenon in biochemistry, synthetic chemists typically cannot reversibly switch on or off catalysts by external effectors.[16−18] This ribozyme may arguably be regarded as a prototype in the development of fully synthetic molecular signaling cascades.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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