Controlling the rate of organic reactions: rational design of allosteric Diels-Alderase ribozymes

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ABSTRACT

Allosteric mechanisms are widely used in nature to control the rates of enzymatic reactions, but little is known about RNA catalysts controlled by these principles. The only natural allosteric ribozyme reported to date catalyzes an RNA cleavage reaction, and so do almost all artificial systems. RNA has, however, been shown to accelerate a much wider range of chemical reactions. Here we report that RNA catalysts for organic reactions can be put under the stringent control of effector molecules by straight-forward rational design. This approach uses known RNA sequences with catalytic and ligand-binding properties, and exploits weakly conserved sequence elements and available structural information to induce the formation of alternative, catalytically inactive structures. The potential and general applicability is demonstrated by the design of three different systems in which the rate of a catalytic carbon–carbon bond forming reaction is positively regulated up to 2100-fold by theophylline, tobramycin and a specific mRNA sequence, respectively. Although smaller in size than a tRNA, all three ribozymes show typical features of allosteric metabolic enzymes, namely high rate acceleration and tight allosteric regulation. Not only do these findings demonstrate RNA's power as a catalyst, but also highlight on RNA's capabilities as signaling components in regulatory networks.

INTRODUCTION

The reversible control of chemical reaction rates by external trigger signals is of great interest from both a fundamental and an application perspective. Catalytic reactions are particularly attractive and nature has evolved elaborate mechanisms of allostery to control enzymatic reaction rates by the concentration of effector molecules. These mechanisms are the basis of feedback inhibition in biochemistry and constitute a central principle of biological regulation (1–3).

Allosteric chemical systems, however, are scarce and limited to a few carefully designed supramolecular and organometallic systems (4–6), mainly owing to our poor understanding of the structural preconditions for switching between catalytically active and inactive conformations of small molecules.

Ribozymes have been converted successfully into allosteric systems by either combinatorial selection or rational design, and both simple oligonucleotides and a variety of other molecules (from metal ions and small organic molecules to peptides and large proteins) were used as effectors (7–11). These catalysts are RNA enzymes whose activity is modulated by effector binding to a domain located apart from the active site (12–15). Although most natural ribozymes catalyze RNA-modifying (i.e. RNA-cleaving or ligating) reactions, ribozymes identified by combinatorial chemistry were found to accelerate a much wider spectrum of chemical reactions, including C-C, C-N, C-O and C-S bond formation or cleavage, and even redox chemistry [for current reviews see (16,17)].

Ligand-induced conformational changes in RNA molecules were discovered recently as an important regulatory principle in gene expression. Riboswitches are metabolite-sensing RNAs typically located in the non-coding portions of messenger RNAs where they control the synthesis of metabolite-related proteins in many species (18,19). More than 2% of the genes in some bacterial species are regulated by riboswitches, and to date, riboswitches have been identified that respond to 10 fundamental metabolites. Typically, a conformational switch in the sensing domain upon ligand binding alters the base-pairing arrangements in the adjoining expression platform, thus modulating transcription, translation or RNA processing events. Recently, a class of catalytic riboswitches was discovered that induce a self-cleavage (i.e. an RNA-catalyzed RNA cleavage) reaction on binding a sugar phosphate metabolite (20).

Phosphodiester chemistry constitutes only for a very small fraction of all biochemically relevant reactions, and the excellent predictability of catalyst–substrate interactions in RNA-catalyzed RNA cleavage reactions clearly sets these systems apart from typical protein enzymes. This latter feature not only allows the rational design of sequence-specific nucleases by the non-specialist, it also facilitates the incorporation of regulatory modules into the same molecule; both extremely

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ambitious tasks in the context of a protein design project. Therefore, allosterically controlled ribozymes that catalyze reactions involving small-molecule substrates would arguably more closely resemble nature's allosteric enzymes and would allow a direct comparison of the regulatory strategies and the underlying structural principles. From an application perspective, such catalysts would offer opportunities to control the rates of chemical reactions for which neither natural enzymes nor synthetic catalysts exist.

Our lab has discovered previously a ribozyme that catalyzes the formation of C–C bonds by Diels-Alder reaction, a reaction type of fundamental importance in organic chemistry. We have accumulated extensive knowledge on the structure and reaction mechanism of this ribozyme, and very recently the 3D structure was solved by X-ray crystallography (21–26). This high amount of structural information renders the Diels-Alderase attractive for the development of allosteric systems. Although we reported recently the first isolation of an allosterically controlled non-phosphodiester ribozyme by combinatorial selection (27), we now investigate different strategies for rational construction of Diels-Alderase ribozymes that are positively regulated (i.e. switched on) by external effector molecules.

Although one cannot convert an inactive RNA sequence into a catalyst by design, it is quite easy to manipulate a catalytically active sequence to fold into an inactive conformation (7,28,29). For allosteric regulation, active and inactive conformation should be at equilibrium, which is shifted by ligand binding. Using the Diels-Alderase ribozyme as the catalytic platform, we directly attach three structurally different ligand-binding modules without intervening spacer sequences. Weakly conserved sequence elements in both domains are used to create alternative structures and to optimize and fine-tune the relative stabilities of the competing active and inactive structures. The key to allostericity lies in this delicate balance, and the three examples provided below illustrate different ways to modulate the rate of catalytic cycloaddition reactions.

MATERIALS AND METHODS

General

Theophylline, tobramycin and biotin maleimide were purchased from Sigma. Anthracene-deca (ethylene glycol) guanosine monophosphate was provided by Dr R. Fiammengo (30). DNA transcription templates and short RNA oligonucleotides (Bcr/Abl and Abl/Abl) were purchased from IBA Göttingen, Germany. RNA–tether–anthracene conjugates were prepared by enzymatic synthesis (T7-RNA polymerase; Stratagene or MBI Fermentas) in the presence of optimized concentrations of anthracene-deca (ethylene glycol)-guanosine monophosphate as transcription initiator, which allowed the preparation of ribozymes with covalently tethered anthracene (30). Transcription products were purified by PAGE. For radioactive labeling of oligonucleotides, $[\alpha\text{-}^{32}P]CTP$ and $[\gamma\text{-}^{32}P]ATP$ (Amersham) were used.

Ribozyme activity assays

Decay kinetics of the fluorescence of covalently tethered anthracene were monitored by spectrofluorimetry (JASCO).

Exitation, 365 nm (slit width 1 nm); emission, 419 nm (slit width 10 nm). The change in fluorescence (dNFS/dt) translates linearly into change in substrate concentration (d[Antracene]/dt). Reactions were carried out in reaction buffer (final concentrations 30 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$ and 300 mM NaCl) at 25°C. The concentration of ribozyme was varied between 1 and 4 µM. All components were mixed, allowed to incubate for 15 min and the start fluorescence value (100%) was recorded. The reaction was started by the addition of biotin maleimide (final concentration: 25 mM, from a 1 M stock solution in dimethyl sulfoxide). Effector concentrations were 100 µM for theophylline and 10 µM for tobramycin. For the Bcr/Abl system, the stoichiometry of the three components (left fragment:right fragment:mRNA) was 1:1:2. Kinetic fluorescence–time curves of all catalytically active constructs are provided as online Supplementary Data.

RESULTS

Design principles and structural analysis of the systems

The operating principle of the allosteric systems described here is the stabilization of catalytically inactive conformations by direct interference of sequence elements of both domains. On binding the effector, this stabilizing interference is overcome and the RNA refolds into a catalytically active conformation (Figure 1). This approach uses complementary stretches of nucleotides that are either already present in the
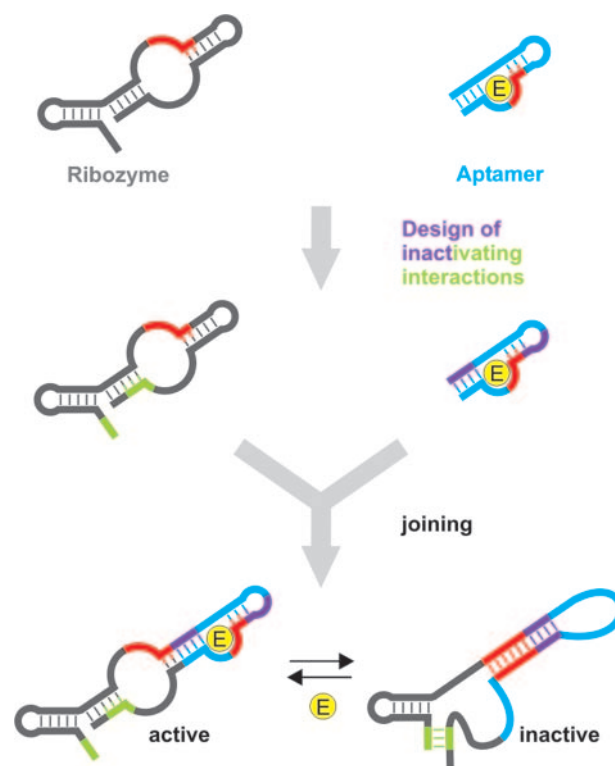

Figure 1. Rational design of allosteric ribozymes operating by direct inactivating interference. An aptamer and a ribozyme are fused, and different interactions are either identified (red) or designed (green, purple) that cause folding into a stable alternative structure. Effector binding induces refolding.
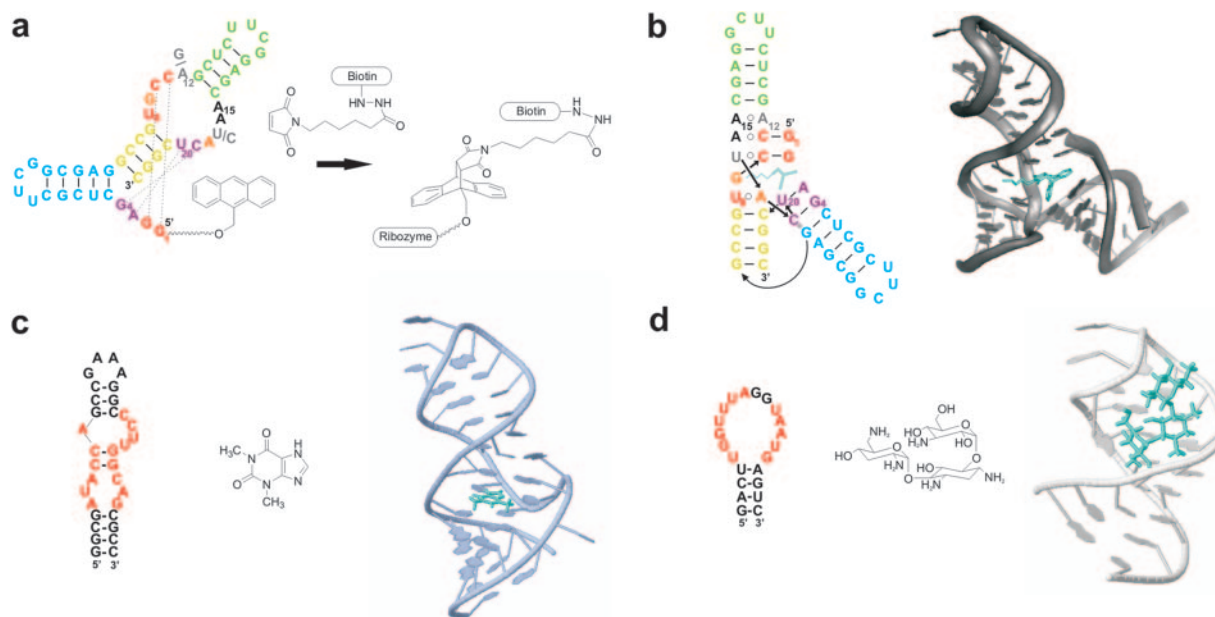
Figure 2. RNA molecules utilized in the design of allosteric ribozymes. (**a**) Secondary structure of the Diels-Alderase ribozyme and catalyzed reaction. Green and blue stem–loop structures are completely variable in size and sequence, while the yellow helix is variable in sequence, but conserved in size. Nucleotides in red are invariant; orange, highly conserved (over 90% activity reduction on mutation); purple, pair-wise complementary substitutions allowed; gray, strong preference for 2 nt; black, variable (<50% activity reduction on mutation); black dotted lines, Watson–Crick pseudoknot base pairs. (**b**) Tertiary structure of the Diels-Alderase ribozyme with the reaction product (cyan) bound inside the catalytic pocket. (**c**) Theophylline aptamer secondary structure (conserved residues in red, variable in black), effector formula and 3D structure with the theophylline in cyan. (**d**) Tobramycin aptamer secondary structure, effector formula and 3D structure with bound tobramycin (cyan).

parental aptamer and ribozyme sequences (original inactivators, red) or can be incorporated at weakly conserved positions by mutation (artificial inactivators, green and purple). In addition to the formation of alternative secondary structures, the approach may also involve the disruption of critical tertiary contacts. Thus, the first step in the development of such systems is the careful analysis of the parental sequences for original inactivators and weakly conserved positions, leading to an initial design. In subsequent steps, the system is gradually modified and optimized.

The reaction catalyzed by the Diels-Alderase ribozyme is shown in Figure 2a, together with the simplified secondary structure. Although this ribozyme is capable of multiple-turnover catalysis, a single-turnover *in cis* reaction format (with the anthracene diene covalently tethered to the RNA) is used throughout this study. The blue and green helices were found to be variable in both size and sequence, while for the yellow helix a length of 4 bp is conserved. The 5′-G1-G2-A3-G4 segment plays a critical role in shaping both the RNA scaffold and the catalytic pocket. All four residues align through Watson–Crick base pairing with residues on both sides of the asymmetric bubble segment (dotted black lines in Figure 2a), thereby generating a complex nested pseudoknot topology and leading to a lambda-shaped overall structure. In Figure 2a, the bases of the 5′-GGAG end and of the asymmetric internal bubble are color-coded according to their conservation level (for details see caption). Figure 2c and d shows the theophylline (31,32) and tobramycin (33,34)

aptamer secondary structures (with the conserved positions in red), their effectors and their 3D structures with the bound effectors shown (in cyan). The various non-conserved or weakly conserved nucleotides found provide ample opportunities for molecular design.

Design of theophylline-responsive Diels-Alderase ribozymes

The sequences of the Diels-Alderase ribozyme and the theophylline aptamer (31) contain a complementary 5 nt stretch (yellow box, Figure 3) that represents the starting point for the design. Ribozyme and aptamer were fused directly by deleting the ribozyme's upper stem–loop and the aptamer's lower stem (Figure 3a). Two artificial inactivators were introduced by mutation, one leading to an alternative aptamer structure (red box) and the other causing the ribozyme's 5′ end hybridizing to its 3′ end (gray box). According to this initial design, three different double-strand interactions (shown as red, yellow and gray boxes) would stabilize the inactive structure, while four stems (indicated by blue, purple, green and red letters) would stabilize the active form.

The activity of all constructs in the absence or presence of theophylline was determined by monitoring the decrease of the anthracene fluorescence at 419 nm as the result of the Diels-Alder reaction (22,27) (Figure 3c). The construct representing the initial design, Theo 1, showed greatly reduced activity, compared to the non-regulated Diels-Alderase ribozyme, but exhibited an 9-fold activation by theophylline (for kinetic curves see Supplementary Figure S1). To improve allostericity we next systematically varied the strength of the
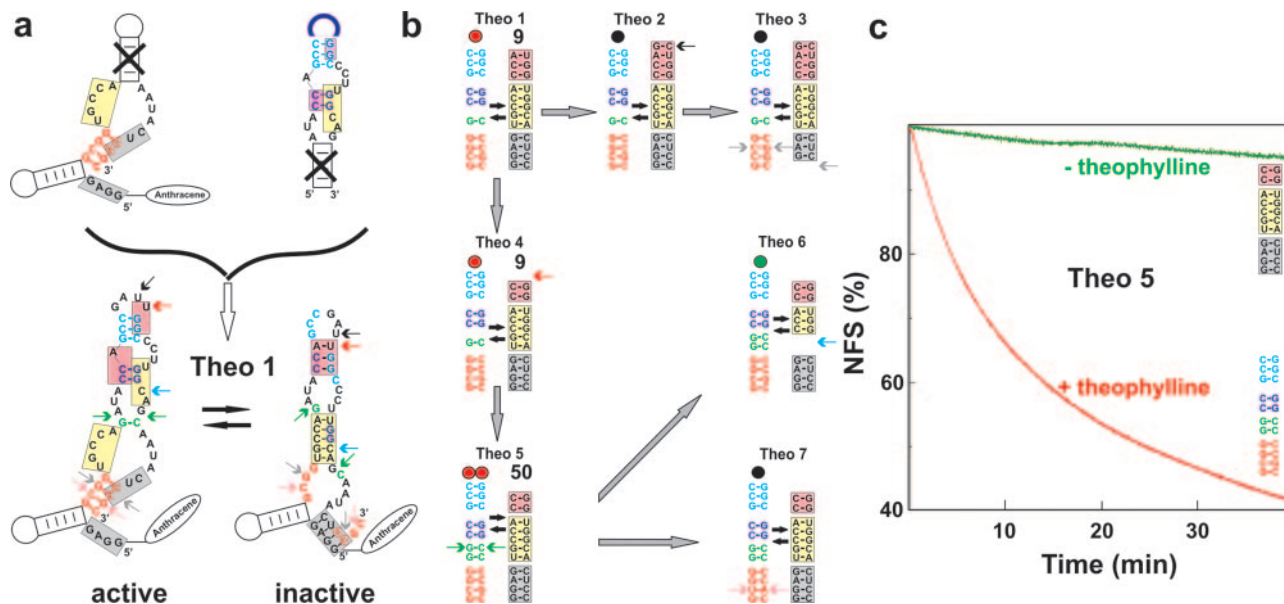
Figure 3. Design of theophylline-dependent Diels-Alderase ribozymes. (**a**) The Diels-Alderase ribozyme (left) and the theophylline aptamer (right) are fused after removing the upper and lower helix, respectively, leading to the starting design Theo 1. Colored boxes indicate interactions that stabilize the inactive structure. Colored letters show interactions that stabilize the active structure. Colored arrows indicate positions of mutations leading to molecules Theo 2 to Theo 7. (**b**) Systematic investigation of interactions that stabilize or destabilize the active (left, colored letters) or inactive (right, colored boxes) structures. The colored arrows indicate the positions of mutations and correspond to (a). Black circle, inactive molecule; green circle, constitutively active molecules; red circle, allosteric molecule; two red circles, good allostericity. Numbers indicate the allosteric switching factor, defined as the initial rate in the presence of effector divided by the initial rate in the absence of effector. (**c**) Fluorescence–time curve for Theo 5 in the presence (red) and absence (green) of theophylline. NFS (%) is normalized fluorescence signal.

different interactions. Stabilization of the inactive structure by lengthening the red box (black arrows in Figure 3a and b) lead to totally inactive construct Theo 2, which could not be reactivated by shortening the gray box (gray arrows, Theo 3). Starting over with Theo 1, we attempted to destabilize the inactive structure by shortening the red box from three to two base pairs (red arrow, Theo 4), which had only marginal effect on the rates. In construct Theo 5, the active structure was additionally stabilized by lengthening the green stem (green arrows), i.e. the direct interface between ribozyme and aptamer domain. This significantly increased the reaction rate in the presence of theophylline and lead to an activation factor of ~50 (Figure 3b and c). Further destabilization of the inactive structure (shortening of the yellow box from 5 to 3 bp, blue arrow) created a constitutively active ribozyme Theo 6 that did not respond to theophylline anymore. On the other hand, a slight destabilization of the active form (by replacing one G–C base pair by A–U starting from Theo 3, pink arrows) gave completely inactive construct Theo 7.

Apparently, Theo 5 provides the optimal balance of stabilities of the active and inactive conformations to allow effector binding to shift the equilibrium towards the catalytically active conformation. The original inactivator (yellow box) is necessary for the proper balance between active and inactive conformation, and mutations that disrupt this element abolish allostericity. The optimal length for the intra-aptamer inactivator (red box) is 2 bp, and stronger inactivators tended to eliminate ribozyme activity. For the stabilization of the active structure, the interface region was found to be particularly important (green letters).

Tobramycin-responsive Diels-Alderase ribozymes

In contrast to the previous system, no suitable complementary stretches exist in the tobramycin aptamer (34), and we have to design a system based solely on artificial inactivators. The main design principle is alternative pairing of the nucleotides forming the two helices above and below the catalytic pocket of the Diels-Alderase ribozyme, which are partially variable (see above, Figure 2a). Thus, in the initial design, Tob 1, the upper stem–loop of the Diels-Alderase was cut off and replaced by the published tobramycin aptamer sequence (Figure 4a). The sequence of the interconnecting stem was designed so that slippage could create an inactive structure with a 5 bp stem (yellow box), involving the CCA of the left side of the catalytic pocket and the terminal U of the right half. The active structure, on the other hand, would be stabilized by two 4 bp helices (blue and red letters). Tob 1 showed some allostericity; the activation factor was about 4.

To increase the stabilities of both the inactive and active structures, one A–U base pair in each structure was replaced by G–C (Tob 2), requiring a total of three point mutations (black arrows in Figure 4a and b), thereby increasing the allostericity to about 9. For both Tob 1 and Tob 2, we next attempted to selectively increase the stability of the inactive structure by increasing the length of the yellow box. The attempt to use 4 (rather than 3) nt of the left side of the catalytic pocket was unsuccessful and lead to completely inactive constructs Tob 3 and Tob 4 (blue arrows). The use of 3 (instead of 1) nt of the right side (red arrows), however, requiring the substitution of C19 by G, and (owing to a known tertiary interaction, Figure 2a and b) a compensatory
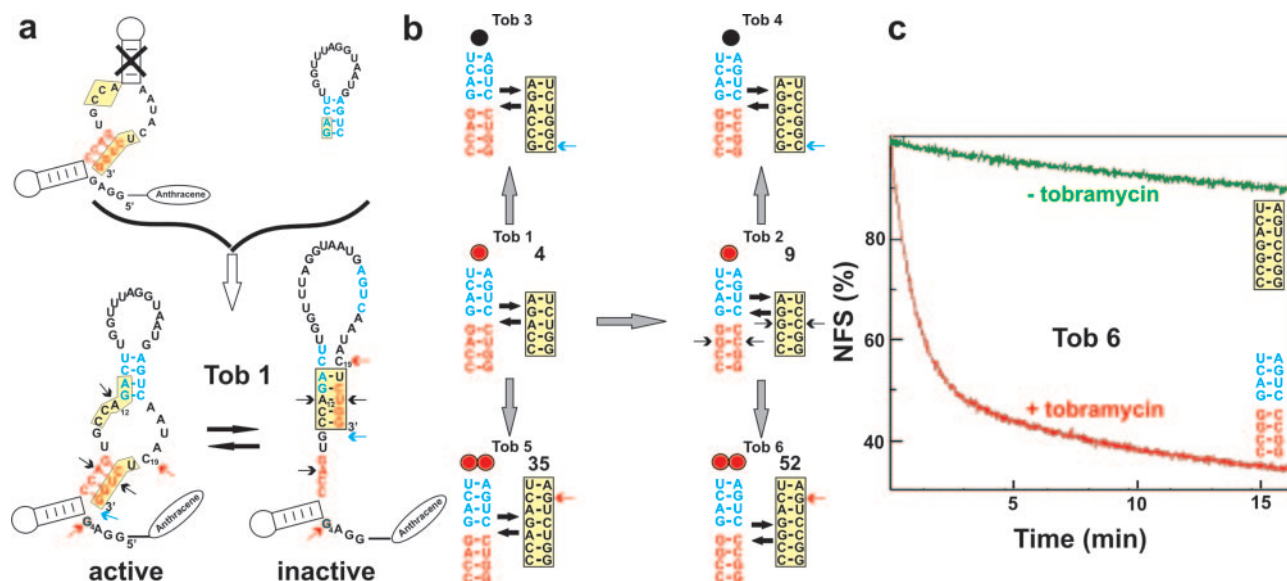
Figure 4. Design of tobramycin-dependent Diels-Alderase ribozymes. (**a**) The Diels-Alderase ribozyme (left) and the tobramycin aptamer (right) are fused after removing the ribozyme's upper helix, leading to the starting design Tob 1. The yellow box indicates interactions that stabilize the inactive structure. Colored letters (red and blue) show interactions that stabilize the active structure. Colored arrows indicate positions of mutations leading to molecules Tob 2 to Tob 6. (**b**) Systematic investigation of interactions that stabilize or destabilize the active (left, colored letters) or inactive (right, colored boxes) structures. The colored arrows indicate the positions of mutations and correspond to (a). Black circle, inactive molecule; red circle, allosteric molecule; two red circles, good allostericity. Numbers indicate the allosteric switching factor, defined as the initial rate in the presence of effector divided by the initial rate in the absence of effector. (**c**) Fluorescence–time curve for Tob 6 in the presence (red) and absence (green) of tobramycin. NFS (%) is normalized fluorescence signal.

mutation of G4 to C, yielded constructs Tob 5 and Tob 6 that showed excellent allostericity with activation factors of 35 and 52, respectively. This result demonstrates that knowledge about the 3D structure is helpful, and in some cases may be required to reach the allosteric design goal.

A Diels-Alderase ribozyme under the control of a Bcr/Abl messenger RNA sequence

In this third example, a three-component allosteric system is created that can be switched on by a fragment of malignant Bcr/Abl mRNA, a chimeric transcription product involved in a genetic disorder causing chronic myeloid leukemia. As an additional requirement, the system should not only be inactive in the absence of the Bcr/Abl mRNA fragment, but also in the presence of an oligonucleotide corresponding to the non-malignant Abl/Abl mRNA. Therefore, a short stretch of nucleotide sequence around the junction of the two exons should be specifically recognized (Figure 5).

A tetranucleotide AAUA occurs both in the Bcr RNA close to the junction point and in the catalytic core of the Diels-Alderase where it is important for the formation of the tertiary structure (yellow box). This feature was used as starting point. A bipartite Diels-Alderase was designed by replacing the major part of the upper Diels-Alderase stem–loop structure by binding arms complementary to the Bcr2/Abl2 fragment around the junction point. As the sequence of this upper stem is variable, we extended the AAUA fragment of the catalytic pocket to CAAUA. Thus, the 3′-GUUAU-5′ fragment of the left binding arm has two potential binding partners; one in the Bcr/Abl mRNA (if present) and one inside the ribozyme's catalytic center.

This design shows excellent allostericity (Figure 5). Only in the presence of the Bcr2/Abl2 RNA effector oligonucleotide, a significant catalytic activity can be measured (red curve). In the absence of effector RNA, the reaction rate is indistinguishable from the uncatalyzed background reaction (black curve). Allosteric activation is at 2100-fold. The Abl1/Abl2 RNA lacking the competing pentanucleotide fragment failed to activate the Diels-Alderase (blue curve). A mutant ribozyme in which the yellow box of the left binding arm was replaced by a non-complementary sequence showed significant residual activity in the absence of effector RNA, highlighting the importance of the stable inactivating interaction (green curve).

DISCUSSION

In this study, we report the first examples of allosteric RNAs that catalyze reactions different from phosphodiester chemistry constructed by a straight-forward rational approach (12). The three examples start from different structural situations and use chemically very different effectors; from a small purine alkaloid bound mainly by stacking (32) to an aminoglycoside antibiotic that is recognized by hydrogen bonds from RNA bases and sugars to the target (33), to a large RNA sequence recognized purely by canonic base pairing. In all three cases, good to excellent allostericity with switching factors of 50–2100 could be achieved. It is noteworthy that in each case, even the initial design displayed significant allostericity (9-, 4- and 2100-fold, respectively). Although higher allosteric activation factors have been reported for some RNA-cleaving ribozymes (9,10), the values
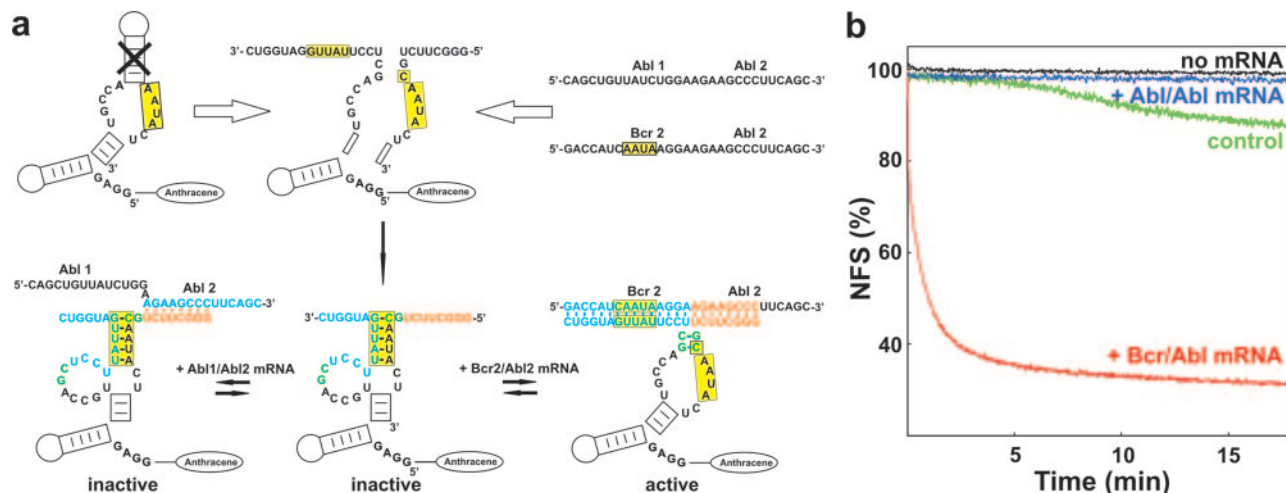
Figure 5. Design of mRNA-responsive Diels-Alderase ribozymes. (**a**) The upper stem–loop structure of the Diels-Alderase ribozyme was replaced by binding arms that specifically recognize the Bcr2/Abl2 mRNA. The yellow box indicates interactions that stabilize the inactive structure. Colored letters (blue, red, green) show interactions that stabilize the active structure. (**b**) Fluorescence–time curves for the different combinations that are shown in (a). The experiment leading to the green curve (control) is identical to that corresponding to the black curve (no mRNA), except that it uses a ribozyme in which the yellow GUUAU box in the binding arm was replaced by CAAUA. NFS (%) is normalized fluorescence signal.

achieved here are in the range of most allosteric protein enzymes in nature (12), and sufficient for the development of sensors and assays (11,35–37). The rationally designed theophylline-dependent ribozyme compares favorably with the one isolated in our lab by *in vitro* selection from a combinatorial library (27).

Rationally designed inactivating interactions have been applied previously to RNA-cleaving ribozymes (most of them under the control of oligonucleotide effectors) (7,28,29,38,39). All these systems capitalize on the predictable nature of canonic base pairing for catalyst–substrate and/or catalyst–effector interaction (13). Here we show that even in cases where Watson–Crick base pairing governs neither of these interactions, it can be used to switch the ribozymes into inactive conformations. Switching efficiency depends on the relative stabilities of the active and inactive conformations, and the delicacy of these equilibria is highlighted by the finding that only in one case (Bcr/Abl) the ribozyme is completely inactive in the absence of effector. In the other two systems, the rate in the absence of effector is still one to two orders of magnitude above the uncatalyzed background reaction (22). Furthermore, several of the kinetic curves show reproducible irregularities and multiphasic behavior, suggesting the involvement of multiple conformations or subpopulations (e.g. Tob 2; Supplementary Data).

The three analytes chosen for this study are all of practical medicinal relevance: theophylline as a brochodilator in asthma therapy, tobramycin as an antibiotic and the chimeric Bcr/Abl transcription product as an important tumor marker. For all three, there is a need for sensitive and robust analytics. The Diels-Alderase generates a directly measurable fluorescence signal and the allosteric constructs allow detecting and determining these analytes (27). The (unregulated) Diels-Alderase was shown previously to act with fast multiple turnovers (up to 7 min^{-1}) on free anthracene substrates, which are very sensitive fluorophores (23). The rational design approach outlined here may therefore allow the

development of sensitive ribozyme-based assays for a variety of analytes without the need for dye-labeled RNA substrate molecules. As the Diels-Alderase was shown to be catalytically active when immobilized (40), surface- and chip-based assays developed for other allosteric ribozymes might work, too (36,41).

The two aptamers and the one mRNA sequence used in this study were selected solely on the basis of published sequence data without prior experiments, indicating that this approach is general with respect to the ligand-binding RNA. It uses general design principles and is probably not restricted to Diels-Alderase ribozymes. We predict that most of the known 'artificial' ribozymes can be rationally put under the control of effectors following these lines, thereby allowing control of the rate of various organic reactions by effector molecules. The only strict requirement is knowledge about sequences and conservation levels for both domains, and additional structural information helps to integrate tertiary interactions into the design.

With sizes under 70 nt, the three ribozymes are even smaller than a tRNA, yet catalyze a demanding organic reaction and they are tightly controlled by their effectors. Not only does this demonstrate the power of RNA as a biocatalyst, but also raises the question whether similar roles are played by RNA in biology. Regulation and signaling are considered to be major biological functions of RNA, and both the currently known riboswitches (18–20) and the recently discovered microRNA based signaling mechanisms appear to be only the tip of an iceberg (42,43). Although these latter mechanisms process information by specific recognition of RNA sequences and subsequent recruitment of proteins, in the systems developed here a small-molecule input signal (the concentration and the chemical identity of theophylline and tobramycin, respectively) or a sequence input signal (in the Bcr/Abl system) is catalytically converted into small-molecule output signal (identity and concentration of Diels-Alder product). We propose that (yet undiscovered)

RNA catalysts for small-molecule chemistry may be involved in biological signaling to integrate RNA and conventional small-molecule signaling.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

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