Rational design of allosteric ribozymes

Jin Tang and Ronald R Breaker

Background: Efficient operation of cellular processes relies on the strict control that each cell exerts over its metabolic pathways. Some protein enzymes are subject to allosteric regulation, in which binding sites located apart from the enzyme's active site can specifically recognize effector molecules and alter the catalytic rate of the enzyme via conformational changes. Although RNA also performs chemical reactions, no ribozymes are known to operate as true allosteric enzymes in biological systems. It has recently been established that small-molecule receptors can readily be made of RNA, as demonstrated by the *in vitro* selection of various RNA aptamers that can specifically bind corresponding ligand molecules. We set out to examine whether the catalytic activity of an existing ribozyme could be brought under the control of an effector molecule by designing conjoined aptamer–ribozyme complexes.

Results: By joining an ATP-binding RNA to a self-cleaving ribozyme, we have created the first example of an allosteric ribozyme that has a catalytic rate that can be controlled by ATP. A 180-fold reduction in rate is observed upon addition of either adenosine or ATP, but no inhibition is detected in the presence of dATP or other nucleoside triphosphates. Mutations in the aptamer domain that are expected to eliminate ATP binding or that increase the distance between aptamer and ribozyme domains result in a loss of ATP-specific allosteric control. Using a similar design approach, allosteric hammerhead ribozymes that are activated in the presence of ATP were created and another ribozyme that can be controlled by theophylline was created.

Conclusions: The catalytic features of these conjoined aptamer–ribozyme constructs demonstrate that catalytic RNAs can also be subject to allosteric regulation — a key feature of certain protein enzymes. Moreover, by using simple rational design strategies, it is now possible to engineer new catalytic polynucleotides which have rates that can be tightly and specifically controlled by small effector molecules.

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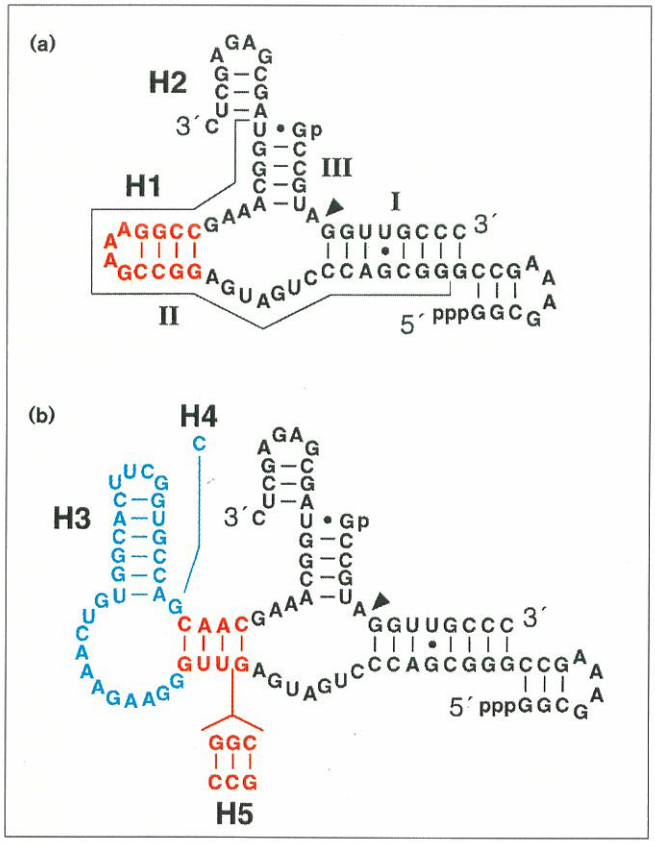
Introduction

The regulation of metabolic processes involves a variety of molecular mechanisms including transcriptional and translational control, RNA and protein degradation, and catalytic induction and inhibition. A number of enzymatic processes are subject to allosteric regulation, in which the catalytic activity of a key enzyme in a metabolic pathway is induced or inhibited upon binding of an allosteric effector molecule. Allosteric regulation of protein enzymes involves allosteric binding sites, located apart from the active site of the enzyme, that can specifically recognize effector molecules. Binding of the effector molecule to the allosteric binding site induces conformational changes in the protein structure that alter the catalytic rate of the enzyme.

Natural ribozymes [1–4] and ribozymes that have been isolated by *in vitro* selection [5–7], are not known to operate as true allosteric enzymes. The activity of natural catalytic RNAs, however, can be greatly affected by specific proteins that support proper structural folding [8–13]. It then seems likely that small-molecule effectors could perform similar functions. Indeed, small antibiotic molecules have been shown both to inhibit and enhance the catalytic rates of certain ribozymes [14–16], although these molecules typically bind directly to the catalytic domain of the RNA and these phenomena are not defined as allostery. Given the significant structural and functional potential for catalytic RNAs [5–7], there would also appear to be significant potential for nucleic acids to function as allosteric enzymes. For example, RNA and DNA aptamers have the capacity to form distinct secondary and tertiary structures that can function as receptors, binding a variety of small organic ligands with considerable affinity and specificity [17–19]. These aptamers can be manipulated as modular receptor elements, a characteristic that has been exploited for the *in vitro* selection of novel ribozymes that make use of pre-existing ligand-binding domains [20,21].

Certain characteristics of ribozyme function can be 'engineered' in a rational fashion by judicious use of simple

Figure 1



Hammerhead ribozyme constructs. **(a)** H1 (shown bracketed) is 'HH15', originally characterized by Fedor and Uhlenbeck [40]. Roman numerals identify stems that form the hammerhead secondary structure. Stem II is also indicated in red. Construct H2 carries an additional G–C base pair in stem I and is flanked on each end by accessory sequences that are designed as short hairpins to reduce the occurrence of inactive structures. **(b)** Construct H3 is an integrated hammerhead ribozyme that includes an ATP-specific and adenosine-specific aptamer (shown in blue and red) [24]. Constructs H4 and H5 are modified versions of H3 that include an aptamer-domain mutation and a three base-pair extension of stem II, respectively. Arrowheads indicate the site of ribozyme-mediated cleavage.

Table 1

Catalytic rates of various ribozyme constructs.

		k_{obs} (min^{-1})		
Construct	Stem II	None	ATP	dATP
H1	AGGCC / AGCCGG	0.58	–	–
H2	AGGCC / AGCCGG	0.10	–	–
H3*	CAAC / GUUG	0.054	0.0003	0.053
H4†	CAAC / GUUG	0.042	0.061	–
H5*	CAAGGCC / GUUCCGG	0.075	0.13	–
H6*	CGUAUGC / GUGUGUG	0.022	0.12	0.027
H7*	CGUGUGC / GUGUGUG	0.0012	0.0098	0.0009

*Constructs containing a functional ATP aptamer. †Constructs containing a defective ATP aptamer. Constructs H6 and H7 are identical to H5 except for the indicated changes in stem II. –, Kinetic values not determined.

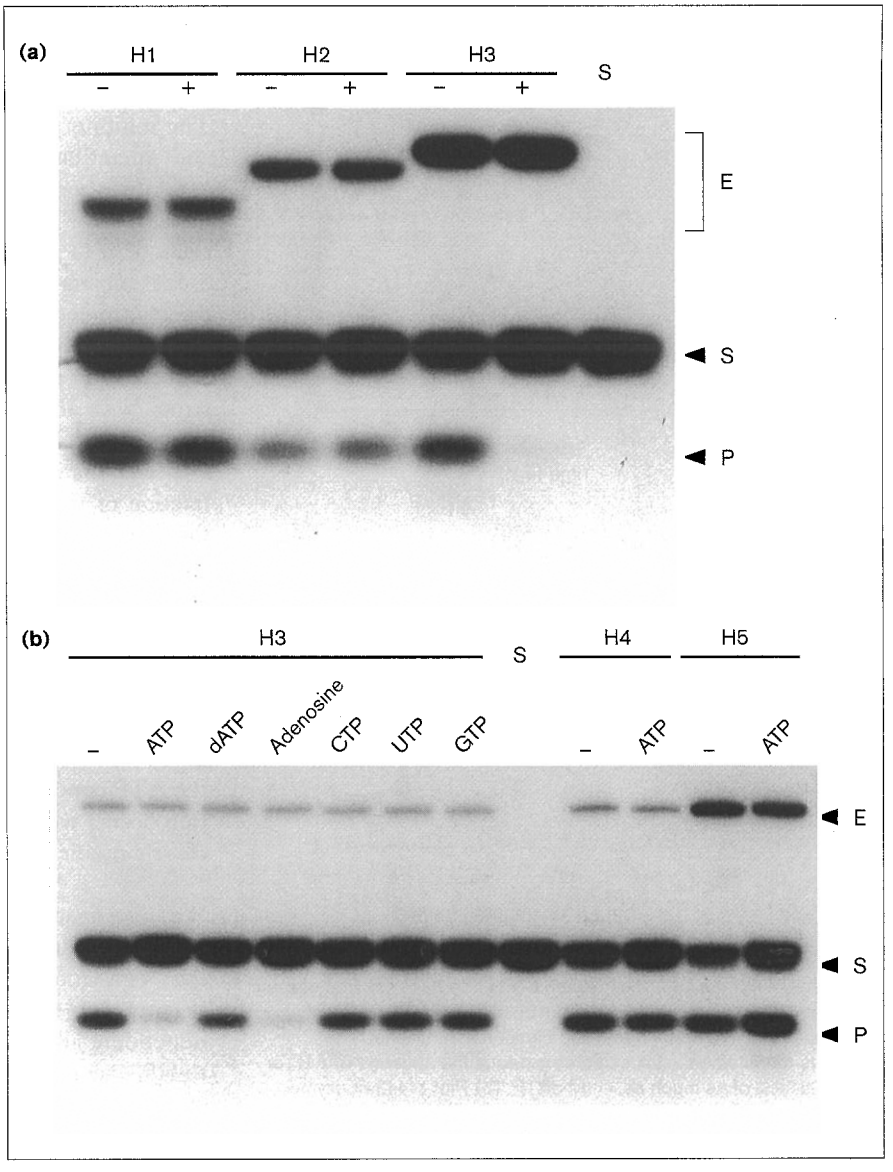
design strategies that make use of Watson–Crick base-pairing rules, thermodynamic stability of RNA duplexes, conformational changes, and the modular use of RNA functional domains [5,6,22]. Here, using these simple rational design concepts, we joined aptamer domains with hammerhead self-cleaving ribozymes [23], to create a series of catalytic RNAs that are amenable either to negative or to positive allosteric control by small-molecule effectors. For our initial work, we used the 40-nucleotide ATP-binding aptamer (ATP-40-1) that was described by Sassanfar and Szostak [24]. The ATP-40-1 aptamer shows specific affinity for adenosine 5′ triphosphate (ATP; K_d ~10 μM) and adenosine, but has no detected affinity for a variety of ATP analogs including 2′-deoxyadenosine 5′ triphosphate (dATP) or the remaining three natural ribonucleoside triphosphates. This aptamer also undergoes a significant conformational change upon ligand binding, as determined by chemical probing studies. We reasoned that these characteristics might be exploited to create a conjoined aptamer–ribozyme molecule that could be subject to ATP-dependent allosteric control.

Results and discussion

We used the bimolecular hammerhead ribozyme construct H1 as a basis for the design of new constructs (Figure 1). First, we appended sequences at the 5′ and 3′ termini to make the new constructs amenable to amplification by reverse transcription–polymerase chain reaction methods for use in subsequent studies, giving construct H2 (Figure 1a) [25]. Construct H2 also differs from H1 in that it has an additional G–C base pair in stem I. These changes cause a sixfold reduction in k_{obs} for H2 compared to that of H1 (Table 1). We next modified stem II to carry the ATP aptamer, giving construct H3 (Figure 1b). Our decision to locate the aptamer here was made primarily because changes in stem II can have large effects on the catalytic rates of hammerhead ribozymes [26,27]. In the absence of

Figure 2

ATP-mediated and adenosine-mediated inhibition of a hammerhead ribozyme. **(a)** Hammerhead constructs H1, H2 and H3 (400 nM) were incubated with trace amounts of [5′–^{32}P]-labeled substrate (S) in the absence (–) or presence (+) of 1 mM ATP for 30 min. **(b)** The specificity of the effector molecule was examined by incubating H3 and S for 45 min as described above in the absence (–) or presence of 1 mM of various nucleotides as indicated. Similarly, constructs H4 and H5 were examined for activity in the presence of 1 mM ATP. Reaction products were separated by PAGE (20%, denaturing) and visualized by autoradiography. E, S and P identify enzyme, substrate and product bands, respectively.



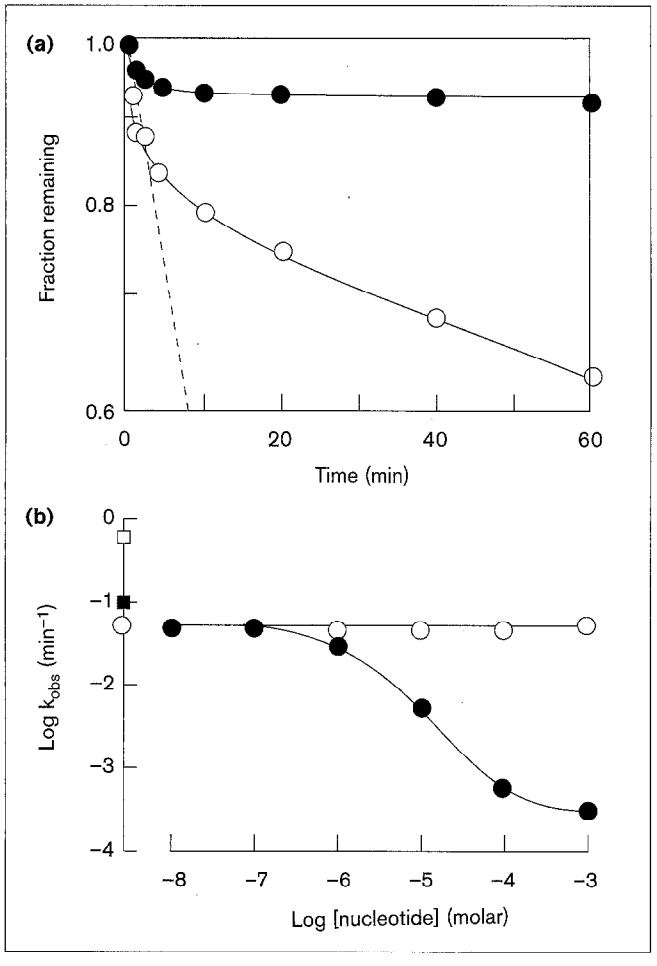
ATP, this alteration in H3 causes an additional twofold reduction in rate compared to that observed with H1.

The RNA-cleavage activity of H3 is significantly reduced when incubated with 1 mM ATP (Figure 2a). In contrast, ATP has no effect on the cleavage activity of H1 or H2. Moreover, inhibition is observed in the presence of adenosine, but not with dATP or the other ribonucleoside triphosphates (Figure 2b). This inhibition is highly specific and is consistent with the previously determined binding specificity of the aptamer [24].

To further investigate the mechanism of inhibition of H3 by ATP, we designed two additional integrated aptamer–ribozyme constructs (Figure 1). Construct H4 is identical to H3, but carries a G→C mutation that disrupts the consensus sequence of the ATP aptamer. This same guanine residue was strictly conserved among the selected ATP-binding RNAs, and a G→A mutation at this position has already been shown to eliminate ATP binding by the ATP-40-1 aptamer domain [24]. As expected, this mutation eliminates the allosteric inhibition effect of ATP with H4 (Figure 2b).

We speculated that the allosteric effect observed with H3 might be due to the close proximity of the aptamer and hammerhead domains. Specifically, structural models of the hammerhead indicate a parallel orientation for stems I and II [28–30]. In the uncomplexed state, the aptamer domain is likely to exist in a single or a set of conformational state(s)

Figure 3



Kinetic analysis of the catalytic inhibition of H3 by ATP. **(a)** Plot of the fraction of substrate cleaved with H3 (400 nM) in the presence of 10 μM (open circles) and 1 mM (filled circles) ATP. Dashed line represents the average initial slope obtained in the absence of ATP or in the presence of as much as 1 mM dATP. **(b)** Plot of H3 ribozyme activity (k_{obs}) in the presence of various concentrations of dATP (open circles) and ATP (filled circles). Also plotted on the y axis are k_{obs} values for H1 (open squares), H2 (filled squares) and H3 (open circles) with no added effector molecules.

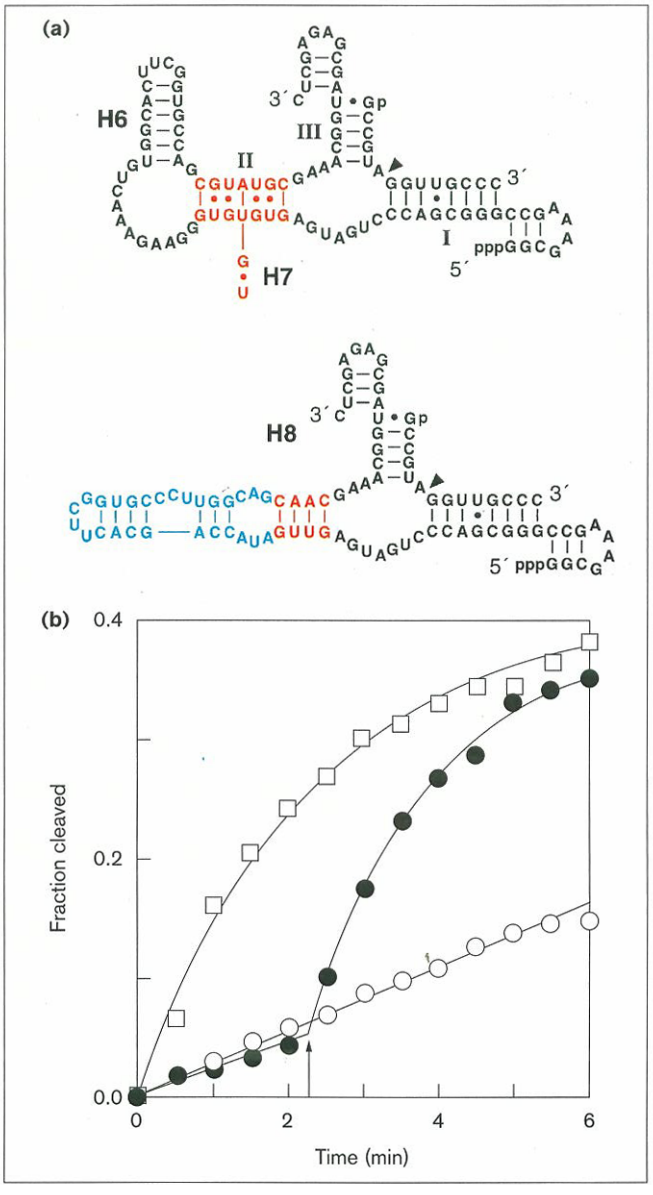
that allow catalysis to proceed unhindered. When complexed with ATP, this domain undergoes a conformational change that presumably causes steric interference between structures that are appended to stems I and II. To test this hypothesis, we designed construct H5 (Figure 1) which has an additional three base pairs in helix II, further separating the aptamer and ribozyme domains. Expansion of stem II from four (H3) to seven (H5) base pairs completely eliminates ATP-dependent inhibition of ribozyme function (Figure 2b). Computer modeling using the recently published structures of the ATP aptamer [31] and the hammerhead ribozyme [28] are consistent with an allosteric inhibition mechanism that involves RNA conformational change and the mutually exclusive formation of aptamer and ribozyme domains (data not shown). We are now proceeding with additional experiments to examine the detailed mechanism of ATP-dependent inhibition of H3 and related aptamer–ribozyme constructs.

The inhibitory effect of ATP with H3 has been confirmed and quantitated by kinetic analyses. This conjoined aptamer–ribozyme construct displays different cleavage rates, after a brief burst phase, with different concentrations of ATP (Figure 3a). The catalytic burst, usually of 3–5% substrate cleavage, was often observed when the effector molecule and the Mg^{2+} cofactor were added simultaneously. This effect was minimized or even eliminated, however, by a 5–10 min preincubation of the RNA construct with the effector molecule prior to initiating the reaction with Mg^{2+}. The catalytic burst seen in the absence of preincubation with effector is probably due to a minor fraction of the ribozyme population that has yet to reach the structurally inactive state prior to reaction initiation. Further evidence that ribozyme catalysis is modulated by ATP binding is obtained by examining the ATP-dependent catalytic rate of H3. A plot of k_{obs} against ATP or dATP concentration (Figure 3b) demonstrates that H3 undergoes ~180-fold reduction in catalytic rate with increasing concentrations of ATP, but is not inhibited by dATP. The ribozyme activity curve generated by varying the ATP concentration indicates that 50% ribozyme inhibition occurs with an ATP concentration near 10 μM, which closely predicts the K_d of the aptamer for its ligand.

We investigated whether ATP could also be made to function as a positive effector of ribozyme function by designing construct H6 and subsequently construct H7 (Figure 4a), both of which were found to display ATP-dependent allosteric induction. The construct H6 is similar to H5, except that four Watson–Crick base pairs in stem II are replaced with less stable G·U mismatches [32]. These changes are expected to weaken stem II significantly and result in diminished ribozyme activity. Here we intended to exploit the fact that the G–C pair that begins stem II within the aptamer domain is not paired in the absence of ATP, but will form a stable pair when ATP is complexed [24], thereby increasing the overall stability of the stem and inducing catalytic activity. Indeed, we find an approximately fivefold reduction in catalytic activity with H6 compared to H5 when assayed in the absence of ATP, yet we can specifically and fully recover H6 catalytic function by the addition of ATP (Table 1). The catalytic rate of H6 is also enhanced by ATP when it is added during the course of the reaction (Figure 4b). Similarly, ATP induces the catalytic rate of H7 by nearly tenfold (Table 1).

Allosteric induction by ATP with H6 and H7 is significantly less than the corresponding ATP-induced rate reduction seen with H3. It is possible that other effects may contribute part or all of the catalytic rate enhancement seen

Figure 4



ATP-dependent allosteric induction of integrated ribozyme constructs. **(a)** Integrated constructs for allosteric induction by ATP (H6 and H7) and allosteric inhibition by theophylline (H8). Stem II is shown in red. H7 differs from H6 in that the central U–A pair is replaced with a G·U mismatch and it is designed to further reduce hammerhead catalysis in the absence of ATP. H8 is analogous to H3 except that the ATP-aptamer domain is replaced by the theophylline aptamer (shown in blue and red) corresponding to 'mTCT8-4' that was described by Jenison *et al.* [38]. Arrowheads indicate the site of ribozyme-mediated cleavage. **(b)** Induction of ribozyme catalysis during the course of a ribozyme reaction was examined by incubating H6 in the absence (open circles) and presence (open squares) of 1 mM ATP, and when ATP is added (filled circles) to a final concentration of 1 mM during an ongoing ribozyme reaction. Arrow indicates the time of ATP addition.

upon the addition of ATP to H6 and H7. As noted above, the addition of the ATP-binding domain results in a twofold drop in ribozyme activity when assayed in the absence of ATP. Perhaps the formation of a defined ATP-binding structure upon the addition of ATP prevents the disordered aptamer domain from incidentally interfering with the function of the ribozyme domain. Further experimentation is necessary to determine the contribution of each structural change towards the sum of allosteric induction.

As with allosteric effectors of proteins, there is no true similarity between the effector molecule and the substrate of the ribozyme. Substrate and effector occupy different binding sites, yet conformational changes upon effector binding result in functional changes in the neighboring catalytic domain. In a previous study, Peracchi *et al.* [33] found that nucleosides could restore the catalytic activity of certain analogs of hammerhead ribozyme that carry abasic sites within their catalytic core. The allosteric ribozymes described in this report are distinct from these examples, in that the binding site for the allosteric effector in our designs lies apart from the catalytic core of the ribozyme, and that the catalytic rates of different ribozyme constructs can either be inhibited or enhanced by effector molecules. The characteristics of the conjoined aptamer–ribozyme complexes parallel the characteristics that are typically ascribed to natural allosteric enzymes that are made of protein. Our proposed model for the mechanism of ATP-dependent control of ribozymes H3, H6 and H7 involves the specific recognition of ATP at a separate binding site that is distinct from the active site, thereby inducing a conformational change that results in the alteration of catalytic rate of the adjacent ribozyme. Specifically, we have preliminary biochemical data that are consistent with the formation of a steric interaction between stem I of the hammerhead and the extraneous stem–loop of the ATP-binding aptamer domain (J.T. and R.R.B., unpublished observations). The putative mechanism, which is identical to that indicated by computer-aided structural modeling, relies entirely on tertiary structure alterations. If correct, this mechanism corresponds well with the original definition for allosteric control of protein enzymes that was originally put forth by Monod and coworkers [34,35].

Using similar model studies, we can now begin to build a palette of design options and strategic approaches that can be used to create ribozymes with controlled catalytic activity. The principles used here (secondary binding sites, conformational changes, steric effects and structural stabilization) as well as others may be generally applicable and can be used to design additional allosteric ribozymes, or even allosteric 'deoxyribozymes' [36,37]. For example, we have designed an allosteric hammerhead that includes the theophylline aptamer described by Jenison *et al.* [38]. This construct (H8, Figure 4a) displays an approximately five-fold reduction in ribozyme activity (k_{obs} of $7.4\times10^{-3}\,min^{-1}$ compared to $1.6\times10^{-3}\,min^{-1}$) when theophylline is added to a final concentration of 100 μM (data not shown). We

are now planning to investigate whether the magnitude of allosteric inhibition or enhancement can be expanded. For example, further revisions of the prototypic allosteric ribozymes described in this report could be made to refine the interplay between the aptamer and ribozyme motifs to improve the net inhibition or enhancement of ribozyme catalytic rates. These improvements can be achieved by making adjustments to the current constructs via rational design, or by constructing a combinatorial library of RNAs followed by screening via *in vitro* selection [7].

The specificity of allosteric control of ribozymes can be exquisite, and in our example the ribozyme activity is sensitive to the difference of a single oxygen atom in the effector molecule. This sophisticated mode of enzyme regulation may have been critical in the 'RNA world' [39], where ribozymes would have had to take advantage of a variety of catalytic and regulatory mechanisms to maintain a complex metabolic state. We have already exploited an H3-like allosteric design to create unimolecular aptamer–ribozyme constructs that can be prepared by *in vitro* transcription without undergoing self-destruction [25]. As with H3, these unimolecular self-cleaving versions of allosteric ribozymes are inhibited during transcription by ATP, which is present as a substrate for RNA polymerase. It is now practical to contemplate the design of a variety of new allosteric ribozymes that are analogous to certain protein enzymes in their ability to be regulated by small-molecule effectors. Allosteric ribozymes could be exploited as rate-regulated catalysts or as 'molecular switches' that could be used to activate or deactivate various biological and chemical processes. Novel biosensors and controllable therapeutic ribozymes, for example, might be of significant importance.

Significance

Using a rational design approach, we have created a series of conjoined aptamer–ribozyme complexes that are subject to allosteric regulation by adenosine and ATP. The conjoined structures can be designed to give either allosteric induction or allosteric inhibition, with catalytic rates in the presence and absence of effector molecule that differ by over two orders of magnitude. These results demonstrate that catalytic RNA molecules can be subject to tight regulatory control, a kinetic feature that increases the potential for RNA to direct sophisticated metabolic processes.

This study has focused primarily on the use of a single ATP-specific aptamer domain and on the use of a single self-cleaving ribozyme. This entire process can most likely be generalized and expanded to create new allosteric ribozymes that have different catalytic functions and that respond to different effector molecules. Growth in the understanding of nucleic acid structure and function will increase the rate at which ribozyme engineers can design new catalysts using rational means, although combinatorial methods can also be used. Considering the propensity of both RNA and DNA to fold into complex shapes, and in view of the increasing number of RNA and DNA aptamers that are being made by *in vitro* selection, it seems reasonable to speculate that a variety of allosteric ribozymes and deoxyribozymes could be engineered for practical applications.

Materials and methods

Ribozyme preparation

Each ribozyme and conjoined aptamer–ribozyme was prepared by *in vitro* transcription from double-stranded DNA templates that were produced by the polymerase chain reaction using the corresponding antisense DNA template and the primers 5′GAATTCTAATACGACTCACTATAGGCGAAAGCCGGGCGA and 5′GAGCTCTCGCTACCGT. Transcription reactions (50 µl) containing 30 pmoles template DNA were incubated in 50 mM Tris-HCl (pH 7.5 at 23°C), 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 2 mM of each NTP, 20 µCi [α–^{32}P]-UTP and 600 units T7 RNA polymerase for 2 h at 37°C. RNA products were separated by polyacrylamide gel electrophoresis (PAGE), visualized by autoradiography and the ribozymes were recovered from excised gel by crush-soaking in 10 mM Tris-HCl (pH 7.5 at 23°C), 200 mM NaCl and 1 mM EDTA and quantified by liquid scintillation counting. The RNA substrate was prepared (Keck Biotechnology Resource Laboratory, Yale University, USA) by standard solid-phase methods and the 2′-TBDMS group was removed by 24 h treatment with triethylamine trihydrofluoride (15 µl per AU260 crude RNA). Substrate RNA was purified by PAGE, isolated by crush-soaking, [5′–^{32}P]-labeled with T4 polynucleotide kinase and [γ–^{32}P]-ATP, and repurified by PAGE.

Ribozyme catalysis assays

Ribozyme activity assays were conducted under single turnover conditions with ribozyme in excess over trace amounts of substrate. Replicate k_{obs} values obtained for H1 and H2 at 200, 400 and 800 nM ribozyme concentration under identical assay conditions differed by less that twofold, suggesting that for each construct, k_{obs} values approach the maximum value. Reactions also contained 50 mM Tris-HCl (pH 7.5 at 23°C) and 20 mM $MgCl_2$, and were incubated at 23°C with concentrations of effector molecules and incubation times as noted for each experiment. Ribozyme and substrate were preincubated separately for ~10 min in reaction buffer and also with effector molecules when present, and reactions were initiated by combining preincubated mixtures. We find that, even after exhaustive incubation with H1, approximately 45% of the substrate RNA remains uncleaved. It is not uncommon for a subset of RNA molecules prepared by chemical means to carry chemical modifications that render the RNA resistant to cleavage, and this probably explains the fact that some substrate remains uncleaved. The kinetic calculations have been adjusted accordingly.

Ribozyme kinetics

Catalytic rates (k_{obs}) were obtained by plotting the fraction of substrate cleaved against time and establishing the slope of the curve that represents the initial velocity of the reaction by a least-squares fit to the data. Kinetic assays were analyzed by PAGE and were visualized and analyzed on a Molecular Dynamics Phosphorimager. When minor-burst kinetics were encountered, a post-burst slope was used in the calculations. Replicate experiments routinely gave k_{obs} values that differed by less than 50% and the values reported are averages of two or more experiments. Equivalent rates were also obtained for duplicate ribozyme and substrate preparations.

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