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Aptazyme-based riboswitches as label-free and detector-free sensors for cofactors

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Abstract—We constructed a label-free and detector-free aptazyme-based riboswitch sensor for detecting the cofactor of the aptazyme. This riboswitch, which usually suppresses the gene expression with its anti-RBS sequence bound to the RBS of its own mRNA (OFF), activates the translation only when a cofactor is added to release the anti-RBS sequence from itself as a result of cofactor-induced self-cleavage by the aptazyme (ON). The rationally optimized one with β-galactosidase as a reporter gene enabled us to detect the cofactor of the aptazyme visibly with high ON/OFF efficiency.

Recently, in vitro-selected RNA or DNA aptamers[1] have been attracting a great deal of attention as sensors for various molecules.[2] Aptamers specifically recognize their targeted small organic molecules, ions, proteins, or cells. Complex formation between aptamers and their targeted molecules results in an increase in mass, which can be directly detected by mass detectors if the target is large.[3] Also, some aptamers cause large structural changes by the binding of target. Such structure-switching aptamers allow us to detect even small molecular targets in combination with fluorescent or electrochemical methods.[4]

'Aptazymes',[5] which are ribozymes that can be activated by particular molecules (cofactors) allosterically, are also able to act as sensors.[6] They contain both an aptamer domain and a ribozyme domain and readily convert the signals of complex formation with their cofactors to more distinct signals of enzyme activities, such as self-cleavage. To date, however, detection of these signals has required that the aptazymes be labeled with radioisotopes[6a] or fluorophores,[6b] or that a special detector such as a quartz crystal microbalance (QCM) be used.[6c] In this study, in order to realize label-free and detector-free detection of the activities exhibited by aptazymes, and thereby to detect their cofactors, we designed an aptazyme-based riboswitch with a reporter gene, which converts the ribozyme activity (self-cleavage) as the complex-forming signal to a more easily detectable signal, that is, expression of a downstream reporter protein in a cell-free prokaryotic translation system.

Our aptazyme-based riboswitch sensor is illustrated in Figure 1.[7] Template DNA was prepared by PCR, wherein an aptazyme sequence was positioned at the 5′-side of the ribosome binding site (RBS) for a reporter gene (luciferase or β-galactosidase) and a short anti-RBS sequence was inserted upstream of the aptazyme sequence. The anti-RBS hybridizes to the RBS to prevent the ribosome from binding to it in mRNA form (OFF state: Fig. 1, bottom left). A stem-loop in the 5′-terminus is required in order to ensure the stability of the transcripts. When transcribing this template in the presence of the cofactor for the aptazyme, the cofactor induces conformational change (I) and the subsequent self-cleavage (II) of the aptazyme. The melting temperature (T_m) of inter-strand duplex is generally lower than that of the intra-strand duplex, so that the cleaved anti-RBS domain will dissociate from the template if the sequence or length of anti-RBS is appropriately chosen (III). Opening the RBS permits the ribosome binding to activate the expression of the reporter gene (IV) (ON state: Fig. 1, bottom right).

As a first attempt, we chose the well-studied theophylline-induced aptazyme, because theophylline hardly

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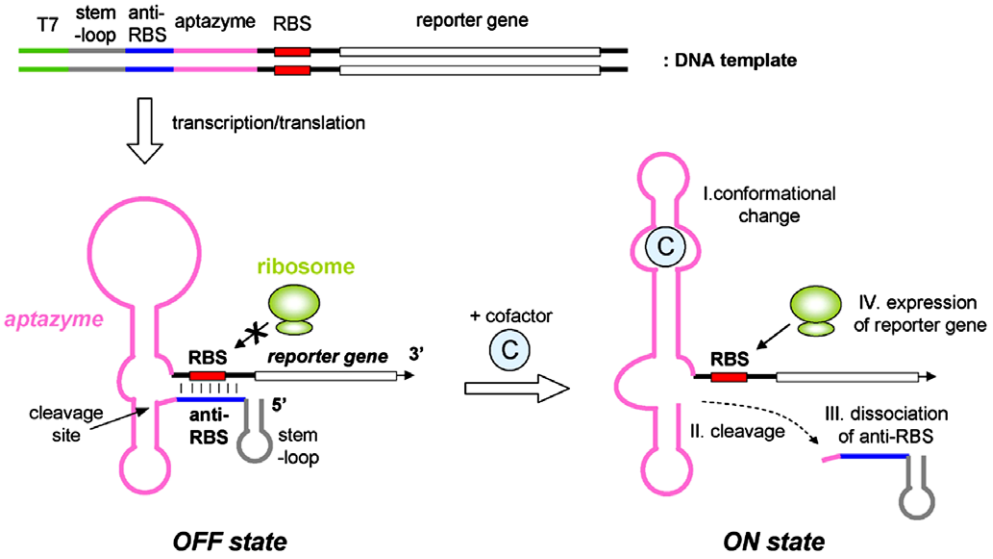

Figure 1. Illustration of an aptazyme-based riboswitch sensor. Template DNA (above) encoding a reporter gene (open bar; luciferase or β-galactosidase) includes a stem-loop (gray), an anti-RBS sequence (blue), an aptazyme (pink), and an RBS sequence (red bar) in this order in the 5′-untranslated region under the T7 promoter (green). When transcribing this template in a cell-free prokaryotic translation system including T7 RNA polymerase, anti-RBS hybridizes to the RBS to prevent the ribosome from binding to it (bottom left; OFF state). In the presence of the cofactor for the aptazyme, the cofactor induces self-cleavage of the aptazyme to activate expression of the reporter gene (bottom right; ON state).

affects in vitro transcription/translation[8] and the corresponding aptazyme has much higher ribozyme activity in the presence of the cofactor (theophylline) than in its absence (1300- to 4700-fold).[5b] This theophylline-dependent aptazyme, which was in vitro-selected by Breaker et al., is composed of three domains: an aptamer, a communication module, and a hammerhead ribozyme domain (Fig. 2a).[5b] We tried to insert a further communication module consisting of an RBS/anti-RBS duplex into helix A or B, since it is known that these helixes have no relation to the activity of this aptazyme.[9] In the case of helix A, the length between the cleavage site and the RBS would be very short, and thus the translation efficiency would likely be low (Fig. 2, inset).[11] On the other hand, in the case of helix B, the above-described length is sufficient (Fig. 2b). In addition, the 5′-terminus of cleaved mRNA retains its stem-loop structure (helix A) so that the mRNA will be stable after the cleavage.[12] Hence, we decided to add the RBS/anti-RBS duplex to the helix B (Fig. 2b).

The sequence and length of the RBS/anti-RBS duplex for high ON/OFF efficiency of translation switching were optimized using luciferase as a reporter gene (luciferase activity was detected by standard luciferase assay; see Supporting Information for details). ON/OFF efficiency is defined here as the ratio of the activity of luciferase translated in the presence (ON) to the absence (OFF) of the cofactor molecule, theophylline. The results are summarized in Table 1. The RBS/anti-RBS duplex **1**, which does not have any bases at the 5′-side of the RBS sequence, was attached just next to GUA triplet (underlined in Fig. 2a) adjacent to the cleavage site.[10] The ON/OFF efficiency of the riboswitch with **1** was 2.2 with luciferase activity of 5708 and 2682 cps in the ON (1 mM theophylline) and OFF state, respectively. This luciferase activity in the ON state was relatively low in comparison with that from a template having a normal 5′-untranslated region (T7 phage g10 leader sequence)[14] instead of the riboswitch under the same conditions (over 100,000 cps). We then inserted the same three purines AAG at the 5′-side of the RBS sequence as present in the T7 phage g10 leader sequence (**2**).[15] The addition of the AAG sequence enhanced the overall translation efficiency by approximately 6-fold in the ON state. On the other hand, the ON/OFF efficiency was improved only ~1.7-fold (ON/OFF = 3.8) due to concomitant expression enhancement in the OFF state. In order to suppress the expression in the OFF state, we then lengthened the anti-RBS to cover the RBS sequence completely with the anti-RBS (**3** or **4**). Indeed, the longer anti-RBS sequence led to the lower translation efficiency in the OFF state. However, the translation efficiency in the ON state was also suppressed, with the result that ON/OFF efficiencies were 4.5 and 4.9 for the riboswitches with **3** and **4**, respectively, which were a little bit higher than that of the riboswitch with **2**. We then introduced a mismatch into the RBS/anti-RBS duplex so that the anti-RBS would cover the RBS with moderate duplex hybridization (**5**). This mismatch resulted in a very high ON/OFF efficiency of 7.2 at (+/−) 1 mM theophylline.[16,17]

Figures 3a and b show ON/OFF efficiencies using the riboswitch with an optimized RBS/anti-RBS duplex **5** at various concentrations of theophylline or caffeine and chemical luminescence images of these translation samples (assay solutions including luciferin), respectively. Although caffeine has a similar structure to theophylline except for the methyl group at the N7 position, the ON/OFF efficiency at (+/−) 1 mM caffeine was nearly equal to 1. These results indicate the high theophylline-dependency and -orthogonality of this riboswitch. Similar experiments using mRNA templates

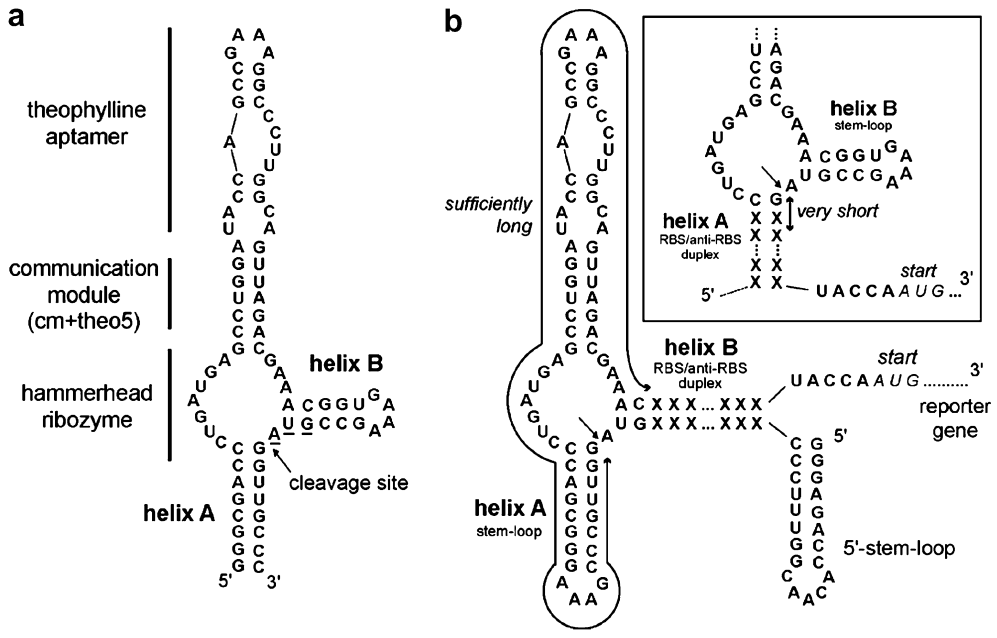

Figure 2. (a) The theophylline-dependent aptazyme in vitro-selected by Breaker et al.[5b] This is composed of three domains, a theophylline aptamer, a communication module, and a hammerhead ribozyme domain. The theophylline cofactor induces a self-cleavage at the cleavage site (arrow) between helix A and B. The GUA triplet adjacent to the cleavage site is underlined. (b) Predicted structure of the designed riboswitches, wherein the RBS/anti-RBS duplex is attached to helix B or A (inset).

Table 1. Luciferase activities and ON/OFF efficiencies in the luciferase assay using various RBS/anti-RBS duplexes

	RBS/anti-RBS duplex	Luciferase activity (cps)		ON/OFF efficiency
	X X X … X X X **X X X … X X X**	— [OFF]	1 mM theophylline [ON]	
1	**AAGGAG**AUA UUUCUCUA	2682 ± 152	5708 ± 472	2.2 ± 0.1
2	AAG**AAGGAG**AUA UUUUUCCU	9087 ± 695	34678 ± 3058	3.8 ± 0.1
3	AAG**AAGGAG**AUA UUUUUCCUUU	1736 ± 165	7827 ± 295	4.5 ± 0.4
4	AAG**AAGGAG**AUA UUUUUCCUUUAUA	690 ± 57	3355 ± 323	4.9 ± 0.3
5	AAG**AAGG^{A}G**AUA UUUUUCC$_{A}$CUAU	4771 ± 275	33675 ± 2905	7.2 ± 0.9

The RBS sequence (AAGGAG) is bold.

instead of DNA templates also showed theophylline-dependency, indicating that this riboswitch is regulated at the translational level but not the transcriptional level (Figure S1).

With the optimized RBS/anti-RBS duplex **5** in hand, we engineered another riboswitch that is controlled by molecules other than theophylline. The aptazyme for cGMP selected by Breaker et al. has high efficiency in ON/OFF switching similar to the theophylline-dependent aptazyme.[5c] Unfortunately, this cGMP-dependent aptazyme includes a sequence complementary to the optimized anti-RBS sequence. We therefore introduced mutations into this complementary sequence so as to retain the ribozyme activity; the mutations were chosen by reference to the literature.[18] However, these mutations were not sufficient to realize precise hybridization of the RBS/anti-RBS duplex, so we used the RBS/anti-RBS duplex **4**, which hybridizes more efficiently than **5** (Figure S2a). The obtained riboswitch has cGMP-dependency with a high ON/OFF efficiency of about 10 at (+/−) 10 mM cGMP (Figure S2b and c). These results showed that it is easy to rationally design other aptazyme-based riboswitch sensors for each cofactor.

Finally, to sense cofactors without the use of a luminometer or other detector, β-galactosidase instead of the luciferase gene was used as a reporter.[7] Figure 3c shows the ON/OFF efficiency at various concentrations of theophylline in a standard β-galactosidase assay. In this assay, translated β-galactosidase hydrolyzes the colorless substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) to yellow *ortho*-nitrophenol, which has sufficient visibility to serve as a marker of this enzyme activity. In fact, just adding ONPG to the solution after coupled transcription/translation (incubated at 37 °C

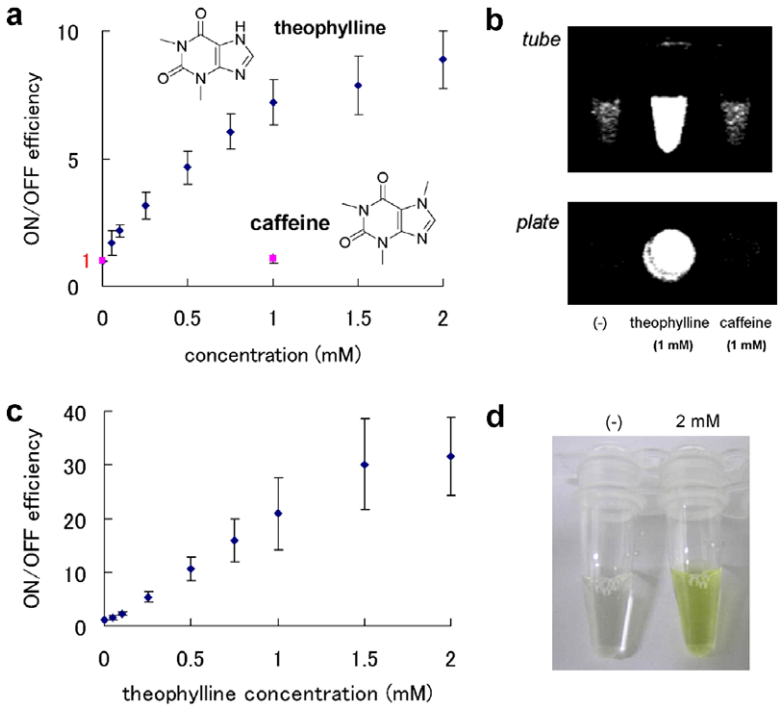

Figure 3. (a) ON/OFF efficiencies in the luciferase assay using a riboswitch with an optimized RBS/anti-RBS duplex **5** at various concentrations of theophylline (blue diamond) or caffeine (pink square). (b) Chemical luminescence images of the luciferase assay solutions in the absence of cofactors (right) or presence of 1 mM theophylline (middle) or caffeine (left). (c) ON/OFF efficiencies in the β-galactosidase assay using an optimized riboswitch with β-galactosidase as a reporter gene at various concentrations of theophylline. (d) Photographic images of the β-galactosidase assay solutions in the absence (left) or presence of 2 mM theophylline (right).

for 1 h) and another incubation at 37 °C for a few hours enables visualization of theophylline without special detectors (Fig. 3d). The ON/OFF efficiencies at (+/−) 1 and 2 mM theophylline, about 20 and 30, respectively, are higher compared to those using the luciferase gene (about 7 and 9).[19]

In conclusion, we constructed aptazyme-based riboswitches with an optimized RBS/anti-RBS duplex and showed their application as sensors for the cofactors of aptazymes without labels or detectors. In this sensor system, stable DNA rather than unstable transcribed RNA can be used as a sensor template in a solution without immobilization or modification. The optimized regulation part (the RBS/anti-RBS duplex) is now available for the design of other aptazyme-based riboswitch sensors with a simple device. The aptazymes used in this study were selected at 23 °C, not at 37 °C, which is the temperature of translation.[5] If aptazymes were selected under proper translational conditions (temperature, sequence of helix B, magnesium concentrations), the ON/OFF efficiency would be higher and sequential problems such as the formation of cGMP-aptazyme would not occur. Further studies along these lines are now underway.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.033.

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Am. Chem. Soc. **2005**, *127*, 5300; (b) Narita, A.; Ogawa, K.; Sando, S.; Aoyama, Y. *Angew. Chem. Int. Ed.* **2006**, *45*, 2879.
8. One millimolar of theophylline did not affect the translation efficiency of luciferase gene with a normal 5′-untranslated region (T7 phage g10 leader sequence).
9. It is noted that duplex formation of the helixes and some bases near ribozyme core are essential to the ribozyme activity.[10]
10. The GUA triplet adjacent to the cleavage site is indispensable to retain the activity of the hammerhead ribozyme; Shimayama, T.; Nishikawa, S.; Taira, K. *Biochemistry* **1995**, *34*, 3649.
11. We separately prepared cleaved mRNA without a riboswitch region, and with a few bases in front of the RBS. The translation efficiency of this cleaved mRNA was very low (data not shown).
12. This is very important because the stability of cleaved mRNA affects the translation efficiency only in the ON state. The 5′-terminus of cleaved mRNA is not triphosphate, which is a 5′-terminal group of general transcripts, but a hydroxyl group that is vulnerable to attack from RNases. However, the contamination by RNases was at a very low level in the reconstituted cell-free prokaryotic translation system used in this study.[13]
13. Shimizu, Y.; Inoue, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Nishikawa, K.; Ueda, T. *Nat. Biotechnol.* **2001**, *19*, 751.
14. The T7 phage g10 leader sequence is commonly used as the 5′-untranslated region to translate the downstream gene efficiently.
15. Cleaved mRNA of **1**-riboswitch without an anti-RBS sequence, which was separately prepared as a template, was not as efficiently translated as the mRNA template having a T7 phage g10 leader sequence (data not shown). These results indicated that the lower expression in the ON state was caused by the unfavorable sequence at the 5′-side of the RBS.
16. Although we attempted to use other mismatched RBS/anti-RBS duplexes by varying the position or number of mismatch, the most efficient of riboswitches with these mismatched duplexes other than **5** had an ON/OFF efficiency of 4.3. As a matter of fact, duplexes **3** and **5** have almost the same melting temperature (estimated by calculations), so that we cannot explain the mismatch effect of **5** with the stability of duplex hybridization. Therefore, at this stage, it is still not clear why this mismatch induces a higher ON/OFF efficiency.
17. We prepared a negative control DNA by introducing a mutation into the GUA triplet of this riboswitch with an optimized RBS duplex **5** (to GUG) to reduce the activity of ribozyme.[10] The ON/OFF efficiency of this mutated one was <1 at (+/−) 1 mM theophylline. This clearly indicated that the self-cleavage of ribozyme is essential for the expression switching.
18. Soukup, G. A.; Derose, E. C.; Koizumi, M.; Breaker, R. R. *RNA* **2001**, *7*, 524.
19. One of the explanations for this difference is that the luciferase gene has more rare codons, which are difficult to read due to the scarcity of the corresponding tRNA.