Riboswitches

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RNA Ligands That Distinguish Metabolite-Induced Conformations in the TPP Riboswitch**

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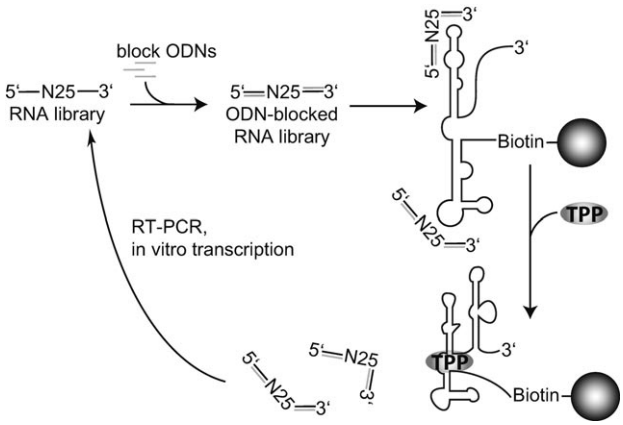
Riboswitches are conserved noncoding elements, located in untranslated regions of messenger RNAs (mRNAs), which regulate the expression of metabolism genes in biosynthetic pathways in response to metabolite binding.[1,2] They bind to small metabolite molecules, thereby triggering the expression of distinct genes that are in turn involved in biosynthetic pathways of the metabolite. As riboswitches regulate the expression of essential genes in many bacteria, these RNAs might serve as targets for the development of novel antimicrobial drugs.[3–5]

The *thiM* riboswitch of *E. coli* regulates the expression of the metabolic protein hydroxyethylthiazolkinase and is known to inhibit gene expression mainly by the sequestration of the Shine–Dalgarno (SD) sequence induced by specific binding of thiamine pyrophosphate (TPP) to an aptamer domain.[6] However, like other metabolite-dependent riboswitches, *thiM* riboswitches also contain an expression domain which does not directly interact with TPP but is required for proper riboswitch function by a mechanism in which TPP-induced structural rearrangements are thought to be relayed to this domain, thereby interfering with gene expression.

Crystallographic and NMR spectroscopy analyses of the aptamer domain of TPP riboswitches in complex with its ligand TPP provided insight into how riboswitch aptamer domains create specific binding pockets.[7–9] However, to understand how these RNAs function, analysis of the complete TPP riboswitch with and without its ligand is required. However, the ligand-free structure is difficult to refine by NMR spectroscopy or crystallography.[10] Therefore, molecular probes that can distinguish between activation states of riboswitches might help to identify sites that are involved in conformational changes and thus, might be useful for gaining insight into the mechanisms of riboswitch function.

One class of molecular probes that can be obtained by in vitro selection are RNA aptamers—single stranded nucleic acids that fold into a distinct 3D-structure and are able to bind with high affinity and specificity to a cognate target molecule.[11] Aptamers have been isolated against a variety of targets including small molecules, peptides, proteins, and living cells[12] and they can discriminate between activation states of proteins.[13] However, no RNA aptamer has been reported to date that is able to differentiate between RNA conformations.

Herein, we describe the isolation of short RNA molecules that are able to bind to the TPP-free *thiM* riboswitch of *E. coli* and are released upon riboswitch–metabolite complex formation. We applied a modified in vitro selection procedure in which the 165-nucleotide (nt) *thiM* riboswitch was biotinylated at its 5′-end and immobilized on streptavidin magnetic beads (Scheme 1). These beads were incubated with a RNA



Scheme 1. In vitro selection scheme for the enrichment of conformation-specific RNA aptamers that bind to the TPP-free *thiM* riboswitch of *E. coli*. Gray sphere = streptavidin magnetic bead; RT-PCR = reverse transcriptase polymerase chain reaction.

library comprising a 25-nt random region. To avoid the participation of the constant regions of the RNA library in *thiM* binding, we sequestered these regions by hybridization to complementary oligodeoxynucleotides (ODNs), leaving the random nucleotides free for independent folding.[14] After removal of all unbound RNA sequences, the retained RNAs were specifically eluted by the addition of TPP (Scheme 1). To favor those RNA species that specifically elute when TPP is present, in selection cycle 7 and 8 we pre-eluted other bound RNAs by the addition of thiamine, which is bound 100-fold less tightly than TPP by the riboswitch. After eight rounds of selection and amplification the resulting RNA

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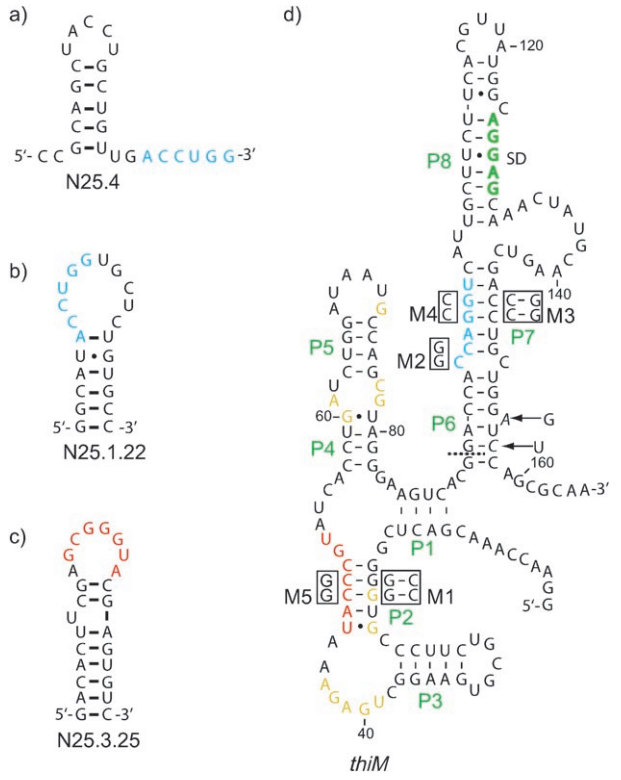
library was cloned and sequenced. The obtained clones can be grouped into one family, sharing an ACCUGG consensus sequence (motif I), located either at the 3′-end (N25.4) or the central part (N25.1) of the random region (Scheme 2 and



Scheme 2. Secondary structure of the initial random region a) of the identified RNA aptamer N25.4, b),c) of the truncated RNA aptamers N25.1.22 and N25.3.25 (see text for details), and d) of the *thiM* riboswitch in its TPP-bound form. Arrows indicate point mutations that allow the cloning of the *thiM* construct for reporter gene assays.[6] The dashed line divides the aptamer and the expression domain. Green nucleotides represent the SD sequence. The sequences shown in cyan and red in (d) represent the complementary regions for motif I (cyan in (a) and (b)) and for the region in red in (c), respectively. The mutants of *thiM* that have been constructed for this study are designated M1–M5. Their sequence modifications are shown in black boxes alongside the wild-type sequences. Nucleotides that are directly involved in TPP binding are shown in yellow.[8] P1–P8 designate the stem regions of the *thiM* riboswitch in its TPP-bound state.

Supporting Information, Figure S1). Among them, clone N25.1 can form a hairpin motif with the consensus sequence in the loop region. Also selected was a unique sequence, N25.3, which forms a hairpin structure with a defined loop region different from the consensus sequence (Scheme 2).

All sequences were analyzed for their ability to bind to *thiM* using surface plasmon resonance (SPR) or electrophoretic mobility shift assays (EMSA; Supporting Information, Figure S2). These analyses revealed dissociation constants K_D of 203 nM for N25.4 and 34 nM for N25.1. Both classes of RNAs bound only in the presence of the blocking ODNs (data not shown), whereas N25.3 still binds equally well in the absence of blocking ODNs with a K_D value of 169 nM. N25.1 and N25.3 can be truncated to 22-nt and 25-nt long RNA hairpins, named N25.1.22 and N25.3.25, that retain their binding properties but lack the binding sites of the blocking ODNs (Scheme 2, Table 1, and Supporting Information, Figure S2). In all cases, the targeting sequences need to be RNA because the corresponding DNA molecules were unable to bind to the *thiM* riboswitch (data not shown).

Table 1: Dissociation constants of aptamer variants determined by surface plasmon resonance.

thiM construct	K_D [M]			
	N25.1	N25.1.22	N25.4	N25.3
thiM (1–165)	3.39×10^{-8}	2.04×10^{-8}	2.03×10^{-7}	1.69×10^{-7}
AD (*thiM* 1–91)[a]	nd[b]	–[c]	nd[b]	2.61×10^{-7}
ED (*thiM* 92–165)[d]	2.14×10^{-7}	–[c]	1.15×10^{-5}	nd[b]

[a] AD: aptamer domain. [b] Not detectable at concentrations up to 1 μM. [c] Not determined. [d] ED: expression domain.

The unpaired consensus sequence in the motif I family is complementary to position 95–101, which belongs to the expression domain of the *thiM* riboswitch (Scheme 2). The unique sequence N25.3 can also be folded into a hairpin motif with the loop complementary to position 46–52 of the aptamer domain (Scheme 2). This complementarity reflects the respective domain specificity; members of motif I bind the expression domain with a K_D one to two orders of magnitude weaker than the full-length riboswitch but did not bind the aptamer domain, and the data for N25.3 indicate no binding to the expression domain but approximately equal binding of the aptamer domain alone and the full riboswitch (Table 1). To confirm that the complementary regions in the respective domains are indeed the targets of the selected hairpins, we performed EMSA assays of riboswitch variants containing mutations in these regions. Mutants M1 in the aptamer domain and M2 or M3 in the expression domain were designed to maintain the overall number of base pairs in P7, but to lose complementarity to the hairpin (Scheme 2). These mutants retained their ability to bind TPP, as quantified by isothermal titration calorimetry (Supporting Information, Figure S3). N25.1.22 still bound efficiently to the aptamer domain mutant M1 but lost the ability to hybridize to M2 and M3, whereas N25.3.25 bound to M2 and M3, but not to M1 (Figure 1), indicating that the hairpin motifs indeed target *thiM* at their complementary sequences in the respective domains.

We performed filter-binding assays to quantify the efficiency by which TPP competes with individual motifs for riboswitch binding and to compare this with thiamine, which is bound approximately 100-fold weaker[6] and should not compete with aptamer binding. Biotinylated riboswitch RNA was incubated with radiolabeled hairpins N25.1.22 or N25.3.25 in the presence of streptavidin and increasing concentrations of TPP or thiamine (Figure 2). N25.1.22 showed half-maximal competition at 1 mM TPP (Figure 2, top), N25.3.25 at 350 μM (Figure 2, bottom), whereas both motifs remained bound to the riboswitch even at thiamine concentrations up to 3.5 mM. Interestingly, the antimicrobial compound pyrithiamine pyrophosphate (PTPP), which

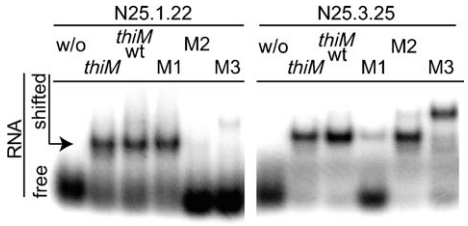


Figure 1. EMSA of N25.1.22 and N25.3.25 with *thiM* and mutants (M1–M3) thereof. The concentrations used were 40 nM in the case of N25.1.22 and 60 nM in the case of N25.3.25. Note: the *thiM* riboswitch used in this study bears two point mutations compared to the wild-type (wt) sequence, namely G155A and U157C indicated by the arrows in Scheme 2.[6] The RNA depicted as *thiM* wt represents the wild-type sequence and was included as another control RNA. w/o = without.

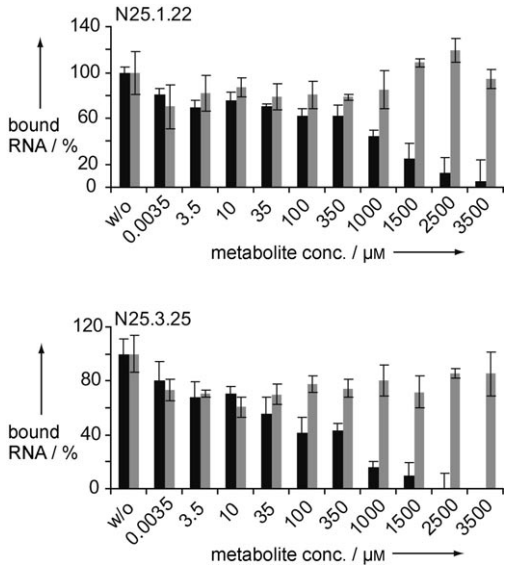


Figure 2. Competition assays of RNA hairpins N25.1.22 (top) and N25.3.25 (bottom) with TPP for *thiM* binding. Black bars: competition with TPP; gray bars: competition with thiamine.

represses the expression of thiamine biosynthesis and import genes,[5] was also able to efficiently compete with both N25.1.22 and N25.3.25 for riboswitch binding (Supporting Information, Figure S5). These observations are in accordance with the notion that the aptamers distinguish between TPP-, or PTPP-induced conformational changes in the riboswitch. For the metabolite-induced release of N25.3.25 from the riboswitch, two mechanisms are possible, either N25.3.25 senses the ligand-free conformation of the riboswitch, or it actively induces conformational changes in the riboswitch aptamer domain that can only be competed with at high TPP concentrations. However, N25.1.22 interacts with the expression domain, therefore the latter mechanism is unlikely. Thus, induction of alternate conformations by N25.1.22 in this domain should not interfere with TPP-binding in the aptamer domain which has been shown to be a self-sufficient TPP binding module.

To test whether the identified sequences that are targeted by the selected RNA hairpins are important for riboswitch function in vivo, we performed reporter-gene assays using *thiM* variants carrying mutations within these regions.[6,15] In mutants M4 and M5 (Scheme 2) the formation of stems P6/P7 and P2, respectively, proposed for the TPP-bound riboswitch, is disrupted. Both mutations caused a loss of genetic control of the corresponding β-galactosidase fusion construct (Figure 3). In contrast, M2 which does not alter the number

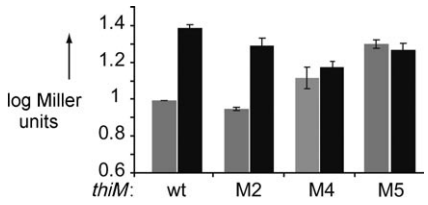


Figure 3. Functional characterization of *thiM* mutants. β-Galactosidase assays were performed in the presence (gray bars) or absence (black bars) of thiamine [100 μM].

of base pairs in P6/P7 (it changes a C–C mismatch to a G–C pair and a C–G pair to a G–G mismatch) does not affect genetic control, indicating that the formation of P6/P7 is as important for riboswitch function as is sequestration of the SD sequence by the anti-SD sequence, thus forming P8. These data confirm that the sequences complementary to our isolated motifs are engaged in the formation of stable helices in the presence of TPP. Conversely, in the absence of the metabolite the same sequences should be accessible for binding to the loop regions in these hairpins.

In conclusion, we applied an in vitro selection scheme and identified RNA aptamers that specifically distinguish the conformations of a functional, metabolite-dependent RNA. The identified RNA motifs bind to either the aptamer or the expression domain of the *thiM* riboswitch. The selected RNA hairpins might have implications for understanding gene regulation through global secondary structure dynamics that the riboswitch undergoes when switching from the TPP-free to the bound conformation. Moreover, the approach introduced herein might facilitate understanding of the function and mechanism of other riboswitches, leading to insights that will be important for their functional engineering, and for further exploration of riboswitches as drug targets. Towards this goal, the results presented in Supporting Information, Figure S5 with PTPP suggest that fluorescence-labeled versions of the TPP-released hairpins identified herein might offer a strategy for the development of high-throughput-screening assays based on FRET (fluorescence resonance energy transfer) or fluorescence polarization[4] to search for compounds that mimic TPP.[5] Such compounds might exhibit antibiotic or antifungal activity by selectively modulating riboswitches in pathogenic microorganisms, a completely unexplored potential target class for new antimicrobial drugs.

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