

Chapter 21

In Vitro Selection of Conformational Probes for Riboswitches

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Summary

Riboswitches are non-coding RNA elements mainly located in the 5' untranslated regions (UTR) of bacterial genes. They bind to small metabolites and upon binding conformational changes occur that trigger the expression of a certain gene. Riboswitches have been identified that bind to amino acids, purines, and other small metabolites such as thiamine pyrophosphate. Riboswitches contain an aptamer domain which is necessary for interaction with the metabolite and a related expression domain which harbours structural and sequence information required for interference with gene expression. The binding of a metabolite to the aptamer domain induces structural rearrangements that are relayed to the expression domain, thereby interfering with gene expression. To investigate and determine domains of the riboswitches which undergo conformational changes upon metabolite binding we used a dynamic SELEX process and identified RNA aptamers that bind to the metabolite-free variant of the riboswitch but are released upon metabolite addition. By this means, and after determination of the binding region, domains which are necessary for proper function of a full-length riboswitch can be identified.

Key words: SELEX, Aptamer, Riboswitch, Kissing complexes

1. Introduction

Thiamine pyrophosphate (TPP)-binding riboswitches represent one of the most intensively studied riboswitch groups (1–7). They can be found in bacteria, regulating either transcription attenuation or translation initiation, and recently also an eukaryotic representative has been investigated and was determined to regulate mRNA splicing (8–10). Amongst the family of TPP riboswitches, the *thiM* riboswitch from *E. coli* was shown to regulate the expression of the enzyme hydroxyethylthiazol kinase by TPP-mediated inhibition

of translation initiation (1, 4). The hydroxyethylthiazol kinase is involved in the biosynthesis and transport of the coenzyme thiamine. The riboswitch exhibits strong TPP-binding activity, and the structure of the aptamer domain of the riboswitch in complex with TPP has recently been solved (11, 12). It is assumed that gene regulation depends on the accessibility of the Shine–Dalgarno (SD) sequence. In the absence of TPP the SD sequence is accessible for the ribosome, whereas TPP binding to the aptamer domain of the riboswitch induces conformational changes in the expression domain of the riboswitch that finally result in the sequestration of the SD sequence, rendering it inaccessible for the ribosome (1, 4, 6). Although riboswitches can be subdivided into an aptamer and an expression domain, both parts of the riboswitch are interconnected by a communication link (4). Upon metabolite binding this module facilitates the transduction of conformational changes from the aptamer- to the expression-domain. Elucidation of these communication links can be accomplished by structural analysis. However, crystallization of full-length riboswitches is difficult to be achieved and so are NMR studies of RNA molecules that bear more than 100 nucleotides in length. A possible alternative approach is offered by the *in vitro* selection of specific RNA molecules that distinguish between two different activation states of a riboswitch (13, 14). For this purpose we developed a specific selection protocol that allows the enrichment of RNA molecules that interact with the TPP-free variant of the *thiM* riboswitch and can be released in the presence of TPP (Fig. 1). The selected RNA molecules were shown to fold into defined short RNA hairpins which form so called kissing complexes with *thiM* riboswitch (4, 13).

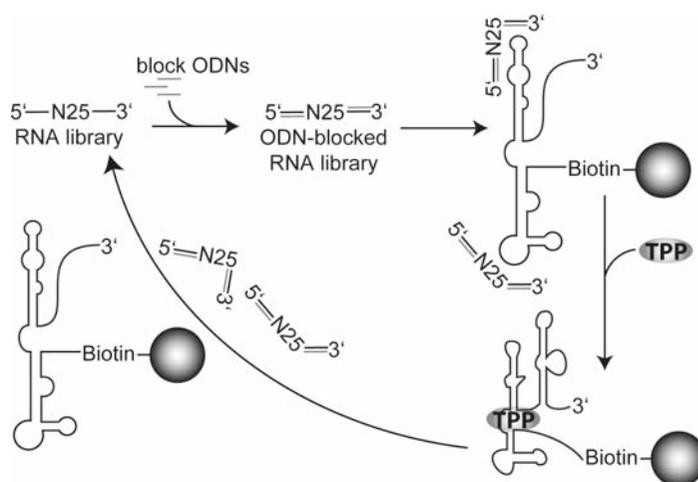


Fig. 1. *In vitro* selection scheme for the enrichment of conformation-specific RNA aptamers that bind to the TPP-free *thiM* riboswitch of *E. coli*. ODN oligonucleotide. Reprinted with permission from Wiley-VCH.

One RNA hairpin was shown to bind tightly to the expression domain and mutational and functional analyses revealed that the binding site of the hairpin is important for the proper function of the *thiM* riboswitch in *E. coli*. This protocol describes the concept and in vitro selection of these specific riboswitch-binding RNA hairpin motifs and the analysis of their binding behaviour by surface plasmon resonance (SPR).

2. Materials

2.1. Oligodeoxynucleotides and RNA Molecules

- Oligodeoxynucleotides were purchased from Metabion, Germany, in 0.04- μ mol scale and HPLC grade. All oligodeoxynucleotides were dissolved in deionized water (ddH₂O) (purified on a Millipore water purification system), and concentrations were determined using UV spectroscopy ($\lambda = 260$ nm).

List of oligodeoxynucleotides:

- 5'-TCGTAATACGACTCACTATAGGAACCAAACGACTCG-3' (5'tpp)
 - 5'-TTGCGCTGGATCCAGCAGGTCTGA-3' (3'tpp)
 - 5'-CGTGACTTCCCTACGCTGGCAT-3' (3'tpp.91)
 - 5'-TCGTAATACGACTCACTATAGACCACCAGGT-CATTG-3' (5'tpp.74)
 - 5'-GGAACCAAACGACTCGGGGTGCCCTTCTGCGT-GAAGGCTGAGAAATACCCGTATCACCTGATCTGGATAATGCCAGCGTAGGGAAGTCACGGACCAC-CAGGTCATTGCTTCTTTCACGTTATGGCAGGAGCAAACCTATGCAAGTCGACCTGCTGGATCCAGCGCAA-3' (tpp.RS)
 - 5'-TGTACCTACGTCTGCAGTGAA-3' (N25.21)
 - 5'-AATACGTAGACTGTCTCTCTCTCCC-3' (5'oligo 1)
- RNA library N25: 5'-GGGAGAGAGAGACAGUCUACGUAUU-N25-UUCACUGCAGACGUAGUACA-3' was obtained by in vitro transcription from the corresponding dsDNA template.
 - Absolute ethanol.

2.2. Denaturing Polyacrylamide Gel Electrophoresis

- Running buffer TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA (*see Note 1*).
- RNA loading buffer: 50 mM EDTA of pH 8.0, 9 M urea.
- 25% Acrylamide/bis solution (37.5:1) in D (this is a neurotoxin when unpolymerized and care should be taken not to receive exposure), Solution D: 8.3 M urea, Solution B: 10 \times TBE in 8.3 M urea.

4. *N,N,N',N'*-Tetramethyl-ethylenediamine (TEMED) (Calbiochem, San Diego, CA) and ammonium peroxodisulphate (APS) (Merck, Darmstadt, Germany).
5. Gel electrophoresis chamber (30 cm × 30 cm) (Fisher Scientific, Pittsburgh, PA).
6. UV handlamp with $\lambda = 254$ nm and silica gel plate with a fluorescing dye (Merck, Darmstadt, Germany).

2.3. Biotinylation of RNA

1. Solid and dried guanosine-5'-thiophosphate (GMPS) (emp Biotech, Berlin, Germany) was dissolved in pure water and its concentration was determined by UV spectroscopy ($\lambda = 260$ nm, $\epsilon = 13,700$ L/mol/cm).
2. Transcription buffer: 20 mM Tris-HCl, pH 7.9, 25 mM MgCl₂, 5 mM DTT, 2.5 mM each NTP.
3. T7 RNA polymerase, 50 U/ μ L (Stratagene, La Jolla, CA).
4. Inorganic pyrophosphatase (Boehringer, Ingelheim, Germany).
5. A spatula tip of EZ-Link PEO-iodoacetyl biotin (Pierce, Rockford, IL) was freshly dissolved in dimethylformamide (DMF).
6. Reaction buffer: 10 mM Tris-HCl, pH 8.0, 50 mM EDTA.
7. RNasin, 40 U/ μ L (Promega, Madison, WI).
8. Phenol saturated with Tris-HCl, pH 8.0 (Roth, Karlsruhe, Germany).
9. Nucleotide triphosphate (NTP) mix, 25 mM each (Larova, Teltow, Germany).
10. NaOAc: 3 M sodium acetate adjusted to pH 5.4 by acetic acid.
11. NH₄OAc: 6 M NH₄OAc, pH 7.4.
12. G25 microspin columns (General Electric, Munich, Germany).

2.4. Magnetic Particle Preparation

1. Magnetic streptavidin-coated Dynabeads, 10 mg/mL (Invitrogen, Carlsbad, CA), were washed 5 times with coupling buffer prior to use.
2. Magnetic particle concentrator (Dynal, Oslo, Norway).
3. Head-over-tail shaker.
4. Coupling buffer: 50 mM Hepes-K, pH 7.5, 100 mM KCl, 0.5 M NaCl, 1 mM EDTA.

2.5. In Vitro Selection

1. Selection buffer: 50 mM Hepes-K of pH 7.5, 100 mM KCl, 5 mM MgCl₂
2. Elution buffer: Coupling buffer supplemented with 5–50 μ M thiamine pyrophosphate (TPP).
3. RT-PCR: One tube set up using superscript II reverse transcriptase (Invitrogen) and Taq DNA polymerase (Promega). For 100- μ L reaction, mix 4 μ L 5 \times First Strand buffer

(Invitrogen), 10 μL 10 \times PCR buffer (Promega), 2 μL 100 mM DTT, 1 μL 100 μM 5'-primer, 1 μL 3'-primer 100 μM , 6 μL 25 mM MgCl_2 , 3 μL 10 mM dNTP mix, 1 μL Superscript (200 U/ μL) polymerase, 1 μL Taq polymerase (5 U/ μL), template, and water to 100 μL .

4. Glycogen dissolved in water.

2.6. Surface Plasmon Resonance Analysis of RNA–RNA Interactions

1. SA sensor chips (Biacore, Uppsala, Sweden).
2. SA-coupling buffer: 50 mM Hepes-K, pH 7.5, 100 mM KCl, 0.5 M NaCl.
3. Running buffer: 50 mM Hepes-K of pH 7.5, 100 mM KCl, 5 mM MgCl_2 .
4. Regeneration buffer: 12.5 mM EDTA, pH 8.0.

3. Methods

3.1. Oligodeoxynucleotides and RNA Molecules

1. RNA molecules for the in vitro selection process, namely the 165 nucleotide containing *thiM* riboswitch and the RNA library N25, were prepared by in vitro transcription from dsDNA templates using standard T7 RNA polymerase-based transcription protocols (*see Note 2*). For 100- μL reaction, mix the DNA template, transcription buffer, 5 μL T7 RNA polymerase, 1 μL RNasin and inorganic pyrophosphatase (optional).
2. The dsDNA templates were performed by common PCR methods using the templates (tpp.RS) and primers (5'tpp, 3'tpp) from **Subheading 2**, whereas the 5'-primer included the T7 promoter region (5'-TAATACGACTCACTATA-3').
3. Purification of dsDNA molecules was achieved by phenol/chloroform extraction, followed by precipitation with ethanol. After centrifugation the pellets were washed with 70% ethanol and dissolved in pure water.

3.2. Denaturing Polyacrylamide Gel Electrophoresis

1. The RNA molecules were purified using denaturing polyacrylamide gel electrophoresis. The glass plates were cleaned carefully with water and ethanol prior to use.
2. Prepare a 1.5-mm thick gel, 10% gel by mixing 28 mL of 25% acrylamide/bis solution, 35 mL solution D and 7 mL solution B, 28 μL TEMED, and 560 μL APS. After pouring the gel insert the comb and use clamps to fix the comb and let the gel polymerize for 30 min.
3. Assemble the electrophoresis chamber and fill the tanks with the running buffer TBE. Remove the comb carefully and rinse the wells using a syringe.

4. Let the gel pre-run about 30 min at 370 V.
5. Dissolve the RNA pellets after transcription work up (*see Subheading 3.3*) in the RNA loading buffer. Heat the samples to 80°C for 1 min and spin in a microcentrifuge to collect the entire sample at the bottom of the tube. Carefully load the well of the pre-run gel and let the gel run for 1.5 h at 370 V.
6. Disassemble the chamber and cover the gel with a thin foil. Place the gel on a fluorescing silica gel plate and use UV irradiation to visualize the corresponding RNA band. Cut the band out and do the work up according to the crush-and-soak method. Finally, the concentration of the purified RNA is measured by UV spectroscopy.

3.3. Biotinylation of RNA

1. Prepare a standard 100 μL in vitro transcription reaction with a 4-fold molar excess of GMPS (10 mM) over GTP (2.5 mM) (*see Note 3*). Incubate overnight at 37°C.
2. For work up of the GMPS-transcription reaction perform a phenol/chloroform extraction followed by ethanol precipitation. Add 100 μL phenol, vortex thoroughly, and spin for 3 min at $17,900 \times g$ in a microcentrifuge. Take the supernatant and add 1 volume of chloroform. Vortex and centrifuge the mixture again. Recover the supernatant, add 30 μL NaOAc and 390 μL ethanol, incubate at -80°C for 10 min, centrifuge at $17,900 \times g$ for 20 min, and remove the supernatant carefully. After washing the pellet with 70% ethanol, dissolve the pellet in 100 μL ddH₂O. Remove excess of GMPS by filtration through G25 microspin columns two times.
3. The GMPS-RNA is then biotinylated by incubation with a 200-fold excess of EZ-link PEO-iodoacetyl biotin in the reaction buffer for 2 h at 40°C.
4. After completion of the reaction, add 1 volume of 6 M NH₄OAc of pH 7.4 and 3 volumes of ethanol and centrifuge for 30 min at $17,900 \times g$. Remove the supernatant, wash the pellet with 70% ethanol, and dissolve the dry pellet in the RNA loading buffer.
5. The biotinylated RNA can be purified by denaturing polyacrylamide gel electrophoresis as described in **Subheading 3.2**.

3.4. Magnetic Particle Preparation

1. Wash 50 μL of streptavidin-coated magnetic beads five times with coupling buffer using magnetic particle concentrator and re-suspend the beads in 250 μL of 2 \times coupling buffer. Add 10 pmol of biotinylated *thiM* riboswitch (250 μL in ddH₂O) and incubate the solution in a head-over-tail shaker for 15 min.
2. Subsequently, the derivatized beads were washed five times with selection buffer and were finally re-suspended in 100 μL selection buffer. The beads were then directly used in the selection process.

3.5. In Vitro Selection

1. Prior to incubation with the *thiM*-bound streptavidin beads, the RNA library N25 should be incubated with the blocking oligonucleotides, which are complementary to the 5'- and 3'-constant primer-binding sites of the RNA library. Prepare a solution containing 50 pmol of the RNA library and 75 pmol of each oligodeoxynucleotide (5'-oligo 1 and N25.21) in selection buffer (380 μ L) without MgCl₂. Heat the solution to 80°C for 3 min and let the solution cool down to room temperature for 15 min. Add 20 μ L 0.1 M MgCl₂ for the proper folding of the RNA library (*see Note 4*).
2. Combine the solution of the *thiM*-derivatized streptavidin beads (**Subheading 3.4, step 2**) with the RNA library solution (**step 1** above) and incubate the reaction for 30 min at room temperature (*see Note 5*).
3. Remove all non-bound RNA molecules by washing 6 times with 100 μ L selection buffer.
4. Elute the bound RNA molecules by the addition of 100 μ L elution buffer containing TPP (*see Note 6*). Incubate for 15 min at room temperature, separate the beads using the magnetic particle concentrator, and remove the supernatant.
5. Precipitate the RNA from the supernatant by the addition of 1 μ g glycogen, 10 μ L 3 M NaOAc of pH 5.4, and 330 μ L ethanol. Incubate at -80°C for 10 min and spin at 20,800 $\times g$ for 10 min. Remove the supernatant, wash the pellet with 70% ethanol, and dry. Dissolve the pellet in 50 μ L ddH₂O and use this solution as template for the RT-PCR.
6. RT-PCR was performed using superscript II reverse transcriptase and Taq DNA polymerase. Reverse transcription was carried out at 54°C for 10 min, followed by inactivation of the reverse transcriptase at 70°C for 15 min. PCR was performed using following settings: 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s, 10–15 cycles (*see Note 7*). All amplified dsDNA products were analysed on 2.5% agarose gels and were subsequently purified by ethanol precipitation. The pellets were dissolved in 20 μ L ddH₂O, and 10- μ L aliquot was used as template for the subsequent in vitro transcription yielding RNA for the next selection cycle.
7. After eight selection cycles, the RNA library was cloned and sequenced (**Fig. 2**). Monoclonal RNA aptamers can be analysed for binding to *thiM* RNA using SPR or electrophoretic mobility shift assays (EMSA).

3.6. Surface Plasmon Resonance Analysis of *thiM*-RNA Aptamer Interaction

1. The streptavidin-coated sensor chip was derivatized with 100 nM biotinylated *thiM*-RNA in SA-coupling buffer by consecutive injections of 5- μ L aliquots of the biotinylated *thiM* RNA (flow rate: 5 μ L/min) until a response of about 1,000 RU was reached (*see Note 8*).

- Interaction of individual RNA aptamers with the *thiM*-derivatized surface was measured by injection of increasing concentrations of the aptamers in running buffer (**Fig. 3**) at flow rate 30 $\mu\text{L}/\text{min}$ (*see Note 9*).
- Regenerate the surfaces by two consecutive injections of the regeneration buffer for 30 s.

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Motif I
#N25.13  CCGCTGCTCCCCGCAGTTGACCTGG
#N25.4   CCGCAGCTACCTGCTGTTGACCTGG
#N25.10  -CCCAGCCGCGAGCTGATGACCTGG
#N25.12  CGACTTTCGTGATGACGTGACCTGG
#N25.9   ACGACGTGTGCACAACATGACCTGG
#N25.8   GTGTGAGTACGCCTCAATGACCTGG
#N25.14  GACGCTTCATCGCGCAATGACCTGG

#N25.1   AGGCATACCTGGTCTCTGTGCCTG

Orphan sequence
#N25.3   CACACTTCGAGCGGGTACGAGTGTG

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Fig. 2. DNA sequences of the selected RNA molecules. Only the initial random region is shown. Sequences can be grouped into two motifs, one unique sequence N25.3 and the motif I. Sequences that are complementary to the *thiM* riboswitch are highlighted in *light* and *dark grey*, respectively. Reprinted with permission from Wiley-VCH.

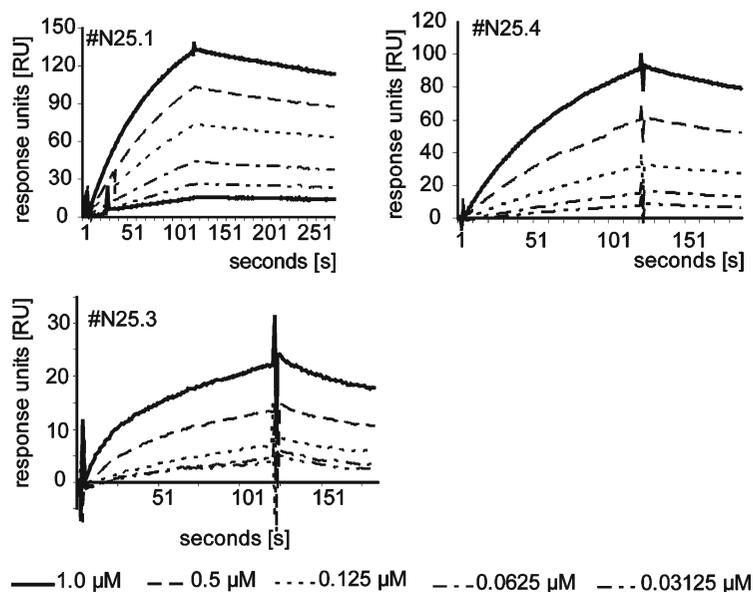


Fig. 3. Surface plasmon resonance analyses of aptamers. SPR analysis of N25.1, N25.4, and N25.3 aptamers. Biotinylated *thiM* RNA was coupled to the streptavidin surface of a CM5 sensor chip and aptamer RNA molecules were injected at indicated concentrations. Regeneration was achieved by 30 s injections with 12.5 mM EDTA, pH 8.0.

4. Notes

1. Avoid nuclease contamination in all buffers and solutions used. Prepare all buffers using DEPC-treated water.
2. Use gloves to avoid nuclease contamination.
3. Avoid oxidation of your GMPS transcriptions by O₂ from the air since oxidation of mercaptane groups hampers the yield of the biotinylation reaction due to disulphide-bond formation of either the GMPS or the GMPS-labelled RNA.
4. Do not heat the RNA molecules in the presence of Mg²⁺ cations since this would favour autohydrolysis of the RNA.
5. The *thiM*-derivatized Dynabeads should be prepared freshly prior to each selection cycle.
6. The amount of TPP used to induce conformational changes of the *thiM* riboswitch and thus to remove bound RNA molecules can vary during the course of selection. Use higher concentrations (50 μM) in the first selection cycles and lower concentrations (5 μM) in the later cycles.
7. Avoid over-amplification of the DNA since this would negatively influence the outcome of the selection.
8. SPR analysis can be done either in the presence or absence of the blocking oligodeoxynucleotides.
9. Make sure that the running buffer used for SPR and the buffer used to dissolve the RNA are identical. Otherwise buffer changes result in worse resolution of the sensograms.

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