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Review

A switch in time: Detailing the life of a riboswitch

Andrew D. Garst, Robert T. Batey *

Department of Chemistry and Biochemistry, 215 UCB, University of Colorado, Boulder, Boulder, CO 80309, USA

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ABSTRACT

Riboswitches are non-protein coding RNA elements typically found in the 5′ untranslated region (5′-UTR) of mRNAs that utilize metabolite binding to control expression of their own transcript. The RNA–ligand interaction causes conformational changes in the RNA that direct the cotranscriptional folding of a downstream secondary structural switch that interfaces with the expression machinery. This review describes the structural themes common to the different RNA–metabolite complexes studied to date and conclusions that can be made regarding how these RNAs efficiently couple metabolite binding to gene regulation. Emphasis is placed on the temporal aspects of riboswitch regulation that are central to the function of these RNAs and the need to augment the wealth of data on metabolite receptor domains with further studies on the full regulatory element, particularly in the context of transcription.

1. Introduction

Bacteria continuously modulate gene expression in reaction to physical and chemical fluctuations in the environment, allowing them to tune their metabolism appropriately to avoid wasteful energy expenditure or inappropriate physiological responses. This formidable challenge employs numerous mechanisms of gene regulation including a large repository of non-protein coding RNAs with diverse biological functions. The regulatory capacity of RNA is readily illustrated by *cis*-acting elements found in 5′-UTRs of bacterial mRNAs that respond to physiological stimuli without the aid of proteins, thereby allowing mRNA to direct its own expression [1,2]. These elements allow mRNAs to directly respond to many discrete environmental cues including changes in temperature, levels of uncharged tRNA, and the concentration of specific metal ions or small molecule metabolites [3]. The primary commonality of all of these regulatory mechanisms, along with some protein-assisted ones, is the presence of a sequence that adopts one of two mutually exclusive secondary structures that lead to expression or repression of the parent transcript [4]. This folding decision occurs cotranscriptionally, with ligand binding steering the RNA into one of two folding pathways that ultimately dictates its fate.

Metabolite sensing RNAs, better known as *riboswitches*, represent a fundamental mechanism of gene regulation that is widespread in the bacterial kingdom; they control ~4% genes in the *Bacillus subtilis* genome alone [5]. In bacteria, they are always found in the 5′-UTR, although examples have been identified in the introns or 3′-UTR in eukaryotic transcripts [6]. There are currently at least twenty classes of riboswitches that recognize a broad range of ligands including purine nucleobases [7,8], amino acids [9,10], vitamin cofactors [11,12], aminosugars [13], metal ions [14], and second messenger molecules [15]. Many "orphan" classes have also been identified using phylogenetic analyses but their metabolites await identification [16,17].

Riboswitches are typically composed of two distinct domains: a metabolite receptor known as the *aptamer domain* and an *expression platform* whose secondary structure signals the regulatory response (Fig. 1). Due to the polarity of transcription, the aptamer domain is transcribed first, providing time for this receptor to sense the cellular environment before the expression platform is fully synthesized. Embedded within the aptamer domain is the *switching sequence* (Fig. 1, red strand), a sequence shared between the aptamer domain and expression platform. The domain this sequence becomes incorporated into dictates which of the two secondary structures the expression domain adopts and thus, the expression fate of the mRNA. Though the term "switch" implies that these secondary structures can interconvert, there is significant evidence that they are typically heavily biased towards a default fold even in the presence of saturating ligand concentrations [18], suggesting that many riboswitches operate more like cotranscriptional fuses.

Although riboswitches provide a relatively simple means of control, these RNAs can be arranged in tandem to generate more complex regulatory responses [4,19]. The simplest form of this involves a tandem arrangement consisting of two riboswitches that respond to the same ligand. The *gvcT* operon for example is controlled by a glycine riboswitch with two aptamer domains that cooperatively bind glycine, a remarkable feature that had only previously been ascribed to proteins [9,20]. Cooperative binding allows a more digital response to small changes in ligand concentration that is unattainable by single copy riboswitches. In other cases the expression response can be coordinated by distinct metabolites, such as that found in the

* Corresponding author. Tel.: +1 303 735 2159; fax: +1 303 492 5894.
E-mail address: Robert.Batey@colorado.edu (R.T. Batey).

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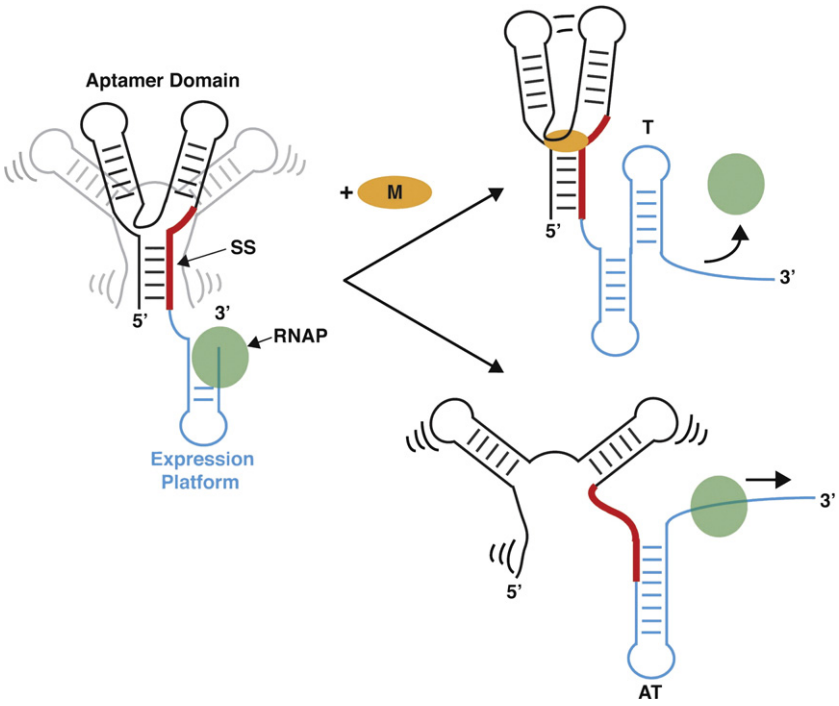

Fig. 1. Riboswitch regulated transcription termination. The aptamer domain (black/gray) forms prior to transcription of the expression platform (cyan), allowing this receptor to sense the concentration of metabolite (orange circle). Metabolite binding collapses an ensemble of the aptamer domain into a single conformation that sequesters the switching sequence (SS) away from pairing with the expression platform. This creates a folding pathway that leads to formation of a terminator stem (T) that causes transcription attenuation. In the event that ligand binding does not occur, particular elements of the aptamer domain remain loosely structured, leading to a conformational switch, that pairs the SS in the expression platform to form the antiterminator stem (AT), allowing RNA polymerase (RNAP) to continue transcribing into the downstream open reading frame.

5′-UTR of the *Bacillus clausii metE* gene that contains both *S*-adenosylmethionine (SAM) and adenosylcobalamin (B12) responsive elements [21]. Each of these riboswitches can independently promote transcription termination upon ligand binding making this mRNA responsive to changes in the concentration of either metabolite. Riboswitches can also overlap with other post-transcriptional control mechanisms. This was recently described for the *Enterococcus faecalis eut* operon, whose expression allows ethanolamine utilization within the human gastrointestinal tract and is thought to affect the virulence of this organism [22]. The operon's polycistronic transcript contains at least one B12 riboswitch that terminates transcription in the presence of coenzyme B12 and many copies of a conserved RNA stem loop that is bound by a response regulatory protein to promote antitermination [22]. In such cases, post-transcriptional regulation rivals the complexity of DNA binding transcription factors that can finely tune gene expression from a specific promoter.

The versatility of riboswitches is further demonstrated by their ability to regulate by a number of means. The most common mode is that of transcription attenuation whereby ligand binding guides formation of a *rho*-independent terminator in the expression platform resulting in transcriptional termination (Fig. 1) [23]. However, there are also several examples of transcriptional "on" switches, such as the *pbuE* riboswitch in *B. subtilis*, that use ligand binding to direct formation of an antiterminator while the default folding pathway leads to termination, demonstrating the modularity of these RNAs [8]. In translational control, riboswitches utilize ligand binding to sequester the Shine–Dalgarno sequence within a helix, thereby impeding association with the 30*S* ribosomal subunit [12]. Additionally, riboswitches can alter the rate of mRNA degradation by directing cleavage or splicing events that promote degradation of the RNA. The *glmS* riboswitch takes a unique approach to this by acting as a ribozyme that uses glucosammine-6-phosphate (GN6P) as an enzymatic cofactor that participates directly in cleavage of the 5′-UTR resulting in destabilization of the transcript [13,24,25]. In contrast, eukaryotic thiamine pyrophosphate (TPP) riboswitches, found in the 3′-UTR or intergenic region of specific thiamine biosynthesis genes, can control alternative splicing of the mRNA by sequestering splice sites within the riboswitch upon ligand binding [6,26].

The discovery of RNA switches in the 3′-UTR of other eukaryotic genes leads to speculation that this region may provide a fertile ground for the discovery of novel mechanisms of riboregulation in these organisms. Indeed, a recently discovered RNA switch in the 3′-UTR of the human VEGFA mRNA forms mutually exclusive structures that alter the production of this growth factor during oxidative stress in myeloid cells [27]. Although this regulatory element is not a true riboswitch, it does adopt mutually exclusive folds that each bind different proteins, each coordinating distinct expression responses [27]. This process is driven by inhibiting proteasomal degradation of one protein during oxidative stress that allows for its intracellular accumulation, thus tipping the balance towards one of the two conformations. Mechanistic details of this process await further investigation, but this study highlights the continuing expansion of our knowledge of the scope of riboregulation in eukaryotic organisms.

2. Structural themes of riboswitch aptamer domains

Many riboswitch regulated pathways are essential to either bacterial survival or virulence, prompting efforts to explore their utility as antibiotic targets [28]. In part, this has motivated efforts to obtain high resolution models of riboswitch–ligand complexes that explain the molecular details of ligand binding and provide clues into how these interactions are coupled to gene regulation. These studies have revealed a number of themes common to the various riboswitches—despite their obvious structural differences—and can be summarized by the recent crystal structure of a lysine-responsive riboswitch (L-box) from *Thermotoga maritima* (Fig. 2) [29,30].

First is the observation that riboswitch architectures are constructed from the same basic recurring tertiary motifs found in other biological RNAs, confirming early predictions that most of the RNA "erector set" has now been observed [31]. For instance, kink-turns,

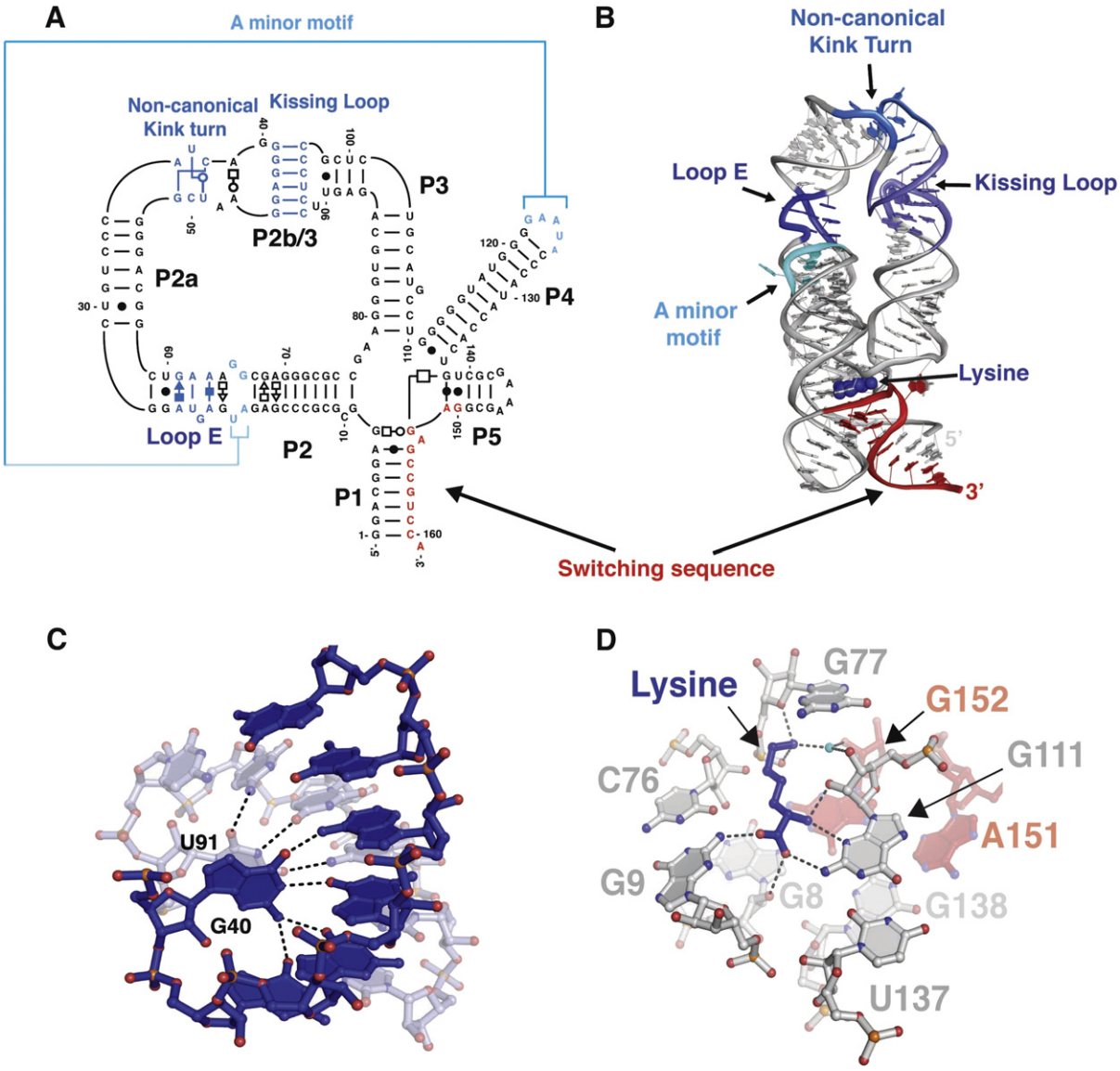

Fig. 2. Structural themes exemplified in the lysine riboswitch from the *asd* gene of *T. maritima*. (A) Secondary structure of the L-box RNA. The non-canonical kink turn motif found in this RNA differs from the canonical K-turn consensus sequence found in other phylogenetic variants of this riboswitch class (L-box consensus). (B) A cartoon representation of the crystal structure of the lysine riboswitch (PDB ID 3D0U). Lysine is bound at the center of the five-way junction, whose fold is facilitated by highly conserved tertiary structures that are highlighted in shades of blue; the mutually exclusive switching sequence is highlighted in red. (C) Close up view of the kissing loop complex formed between L2 and L3. U91 and G40 form a dinucleotide stack that coordinates perfectly with the major groove of this six base pair helix. (D) Hydrogen bonding contacts made between the ligand and the riboswitch illustrate the participation of the switching sequence (red) peripheral to the binding site (A151 and G152). Coloring is the same as panel (B).

ribose zippers, and loop E motifs, all abundant in the ribosome and other large RNAs, introduce bending and long-range tertiary interactions in many riboswitch aptamer domains, including the L-box (Fig. 2A and B), that are essential to their regulatory functions. A classic GNRA tetraloop/tetraloop receptor which is critical to folding of group I introns [32,33] is also conserved in particular sub-classes of the di-cyclic GMP binding riboswitch [15]. The SAM-II and preQ$_1$ riboswitches both conform to classic H-type pseudoknot folds when bound to their respective ligands [34–36], a fold adopted by many viral RNAs that promote frameshifting during translation [37] and a key functional element of telomerase RNA [38]. Surprisingly, even some of the more complex riboswitch architectural modules have been previously observed; the structure of an FMN binding riboswitch contains two symmetrical T-loop domains (P3–P5 and P2–P6) that align with the H19–H20 domain of the *Haloarcula marismorti* 23*S* rRNA with an r.m.s.d. of 1.48 Å, a striking similarity [39].

A second structural trend that encompasses more global aspects of riboswitch folding is the recurrence of helical bundles formed by coaxially stacked Watson–Crick paired helices held parallel to one another by the various motifs mentioned above. In the SAM-I riboswitch for example, two coaxial helical stacks pack together to yield an arrangement similar to the catalytic core of group I introns [40,41]. Like SAM-I, the purine and TPP riboswitches also form two helix bundles, while the glmS, Mg^{2+}, and lysine riboswitches all have a three helix bundle arrangement [2]. In the *T. maritima* L-box helical packing is facilitated by a non-canonical kink turn (Fig. 2A and B) that bends the P2 helix by ~120° to position L2 properly for formation of a kissing loop complex with L3, similar to interaction that promotes genome dimerization in the human immunodeficiency virus [42]. The L-box kissing loop motif is further stabilized by a unique dinucleotide stack that interacts within the major groove of this six base pair L2–L3 helix, a feature that likely promotes thermostability in this RNA (Fig. 2C). The third coaxial stack (P4/P5) is cemented to the P1/P2a stack through an A-minor interaction mediated by two highly conserved adenines in the L4 pentaloop (A123 and A124) docking into the minor groove of P2a adjacent to a canonical Loop E motif [43,44]. Mutations

that disrupt these motifs in the L-box compromise its affinity for lysine and ability to control transcription, demonstrating the linkage between tertiary structure formation and ligand binding [45,46].

A third feature that provides a structural basis for the high affinity and selectivity of riboswitches is the use of binding pockets that typically envelop the metabolite, allowing recognition of nearly every functional group [47]. The L-box uses a five-way helical junction (Fig. 2A) as a binding site that completely encapsulates lysine, coordinating binding through a network of hydrogen bonds to the main chain atoms and an electrostatic interaction with positively charged side chain (Fig. 2D). The length and charge of the side chain account for lysine specificity over smaller amino acids that could easily be accommodated within this pocket. Ornithine, with a side chain that is a single carbon unit shorter than lysine, is unable to promote transcription termination *in vitro* at concentrations >10 mM [10]. Additionally, the L-box makes use of a metal ion (K^+) in the binding pocket to help meditate RNA–ligand recognition [30], a strategy also employed by the TPP [48,49] and FMN riboswitches [50].

Finally, almost all riboswitch structures have revealed that the switching sequence incorporated within the binding site or immediately adjacent, implying an intimate linkage between ligand binding and sequestration of this sequence within the aptamer domain. Indeed, in combination with chemical probing techniques, it is clear that ligand binding is accompanied by conformational changes around the binding pocket that often include the switching sequence. This concept was first uncovered through a structural analysis of the *B. subtilis xpt–pbuX* guanine riboswitch, a transcriptional regulator, in which ligand binding stabilizes formation of two base triples between residues in the three-way junction and the switching sequence [51]. A different solution is adopted by the SAM-II riboswitch where the SAM ligand makes contacts with the Shine–Dalgarno sequence, thereby directly obstructing ribosome binding [34]. Thus the aptamer domain and the expression platform are fully integrated into a single SAM binding pseudoknot. In the L-box, the switching sequence (Fig. 2A, red) forms part of the binding pocket [29]. Chemical and in-line footprinting of the RNA indicate that lysine induces the G8•G152 and G138•A151 pairing interactions (Fig. 2D) that stabilize incorporation of the switching sequence into the aptamer domain [10,29,30,52]. Thus, in every riboswitch that has been structurally characterized to date, ligand binding is directly coupled to conformational changes that involve the switching sequence, which forms the basis for their regulatory activity.

3. Riboswitch free states and ligand induced folding

A comprehensive understanding of riboswitch regulation requires equal consideration of the free-state structure of the aptamer domain. In its unliganded form, the RNA must maintain a conformation (or, more precisely, an ensemble of conformations), in which the binding site is accessible to the ligand. Second, comparison of free and bound state aptamer structures yields atomic-level details about the nature of the conformational changes associated with ligand binding and how these changes lead to regulatory control. Finally, the free state must be able to efficiently direct the RNA along the default folding pathway upon synthesis of the expression platform if ligand binding does not occur, which often requires dissembling part of the secondary structure of the aptamer domain (Fig. 1).

In light of the large structural rearrangements usually depicted by cartoons and secondary structure representations, it may seem surprising that many aptamer domains are highly structured in the absence of ligand, undergoing only local conformational changes upon binding. Riboswitches that fit this description have been classified type I RNAs [2], with the purine riboswitch serving as the current model system. Studies using a variety of biochemical and structural techniques have shown that this RNA is globally organized in the absence of ligand by a conserved loop–loop interaction and only a small number of nucleotides in the highly conserved three-way junction remain loosely structured [7,53,54]. Conformational ordering of these nucleotides occurs only after formation of an initial encounter complex with the ligand. The identity of many of the nucleotides in the binding pocket are crucial not for ligand recognition, but rather maintaining the site in an "open" conformation [18,55]. Chemical probing and mutagenesis revealed that a number of nucleotides around the binding pocket, if altered, lead to a marked loss in the ability to efficiently recognize ligand, despite the fact that the structure of the bound complex is identical to wild type. These studies strongly argue that that there is considerable selective pressure on the sequence of the aptamer domain to maintain ligand-accessible conformations that do not trigger the inappropriate regulatory response [55].

Analyses of the L-box have revealed that this RNA is also a type I riboswitch. Small angle X-ray scattering (SAXS) data show that the L-box undergoes a large compaction upon addition of Mg^{2+} [29], consistent with the requirement of this ion for stabilizing key tertiary elements [56,57]. The addition of lysine however, causes very little observable change in SAXS and native gel electrophoresis experiments, indicating that the ligand induced conformational change of this RNA is limited to local regions, much like the purine riboswitch. Chemical probing of the lysine riboswitch have demonstrated that this change involves a small set of nucleotides found in the five-way junction that form non-canonical pairing interactions in the core of the binding pocket [29] (Fig. 2D). Notably, the crystal structure of the unliganded L-box has also been solved, and is nearly identical to its bound conformation [29,30]. While this certainly does not represent the full conformational ensemble of the free-state RNA in solution, it suggests this conformation is populated to some degree in the absence of ligand. Since the binding pocket is inaccessible to lysine in this conformation, it must exchange with a set of open conformers in the absence of ligand, the rate of which likely has considerable implications on the rate of ligand binding and thereby the regulatory mechanism (*vide infra*).

Other riboswitch aptamers appear to be more unfolded in the free state, and experience global conformational change upon ligand binding, giving them the classification of type II RNAs [41]. These RNAs have bipartite binding pockets that span the coaxial stacks of riboswitches as illustrated by the thiamine pyrophosphate (TPP) riboswitch. In-line probing and limited nuclease digestion demonstrate that ligand binding is coupled to the formation of a GNRA like tetraloop docking interaction between loop 5 (L5) and the P3 helix of this RNA, suggesting that the free state does not adopt the helical packing observed by crystallography [12,48,49]. Fluorescence spectroscopy of RNA labeled site-specifically with 2-aminopurine showed that the L5–P3 interaction forms rapidly upon addition of TPP, serving to orient the P4/P5 coaxial stack in parallel with the P2/P3 stack, nucleating slower formation of the three-way junction and P1 helix that contains the regulatory switching sequence [58]. Thus ligand binding is propagated into distal conformational changes that result in a regulatory response. Interestingly, it has been proposed that folding and binding can be decoupled in this RNA by mutations that retain ligand binding, but prevent folding of a crucial base quadruple in the three-way junction [49].

4. Temporal aspects of riboswitch regulation

Since the early recognition that riboswitches are modular RNAs [12], most structural studies have focused on aptamer domains in isolation, providing only a snapshot in the life of a riboswitch. However, it is important to recognize mRNA folding in the context of transcription is central to the regulatory function of these RNAs. During each individual transcription event, the aptamer domain must first be able to rapidly fold into an active conformation with high fidelity (the "aptamer" time regime; black, Fig. 3) and bind to its effector ligand or undergo a

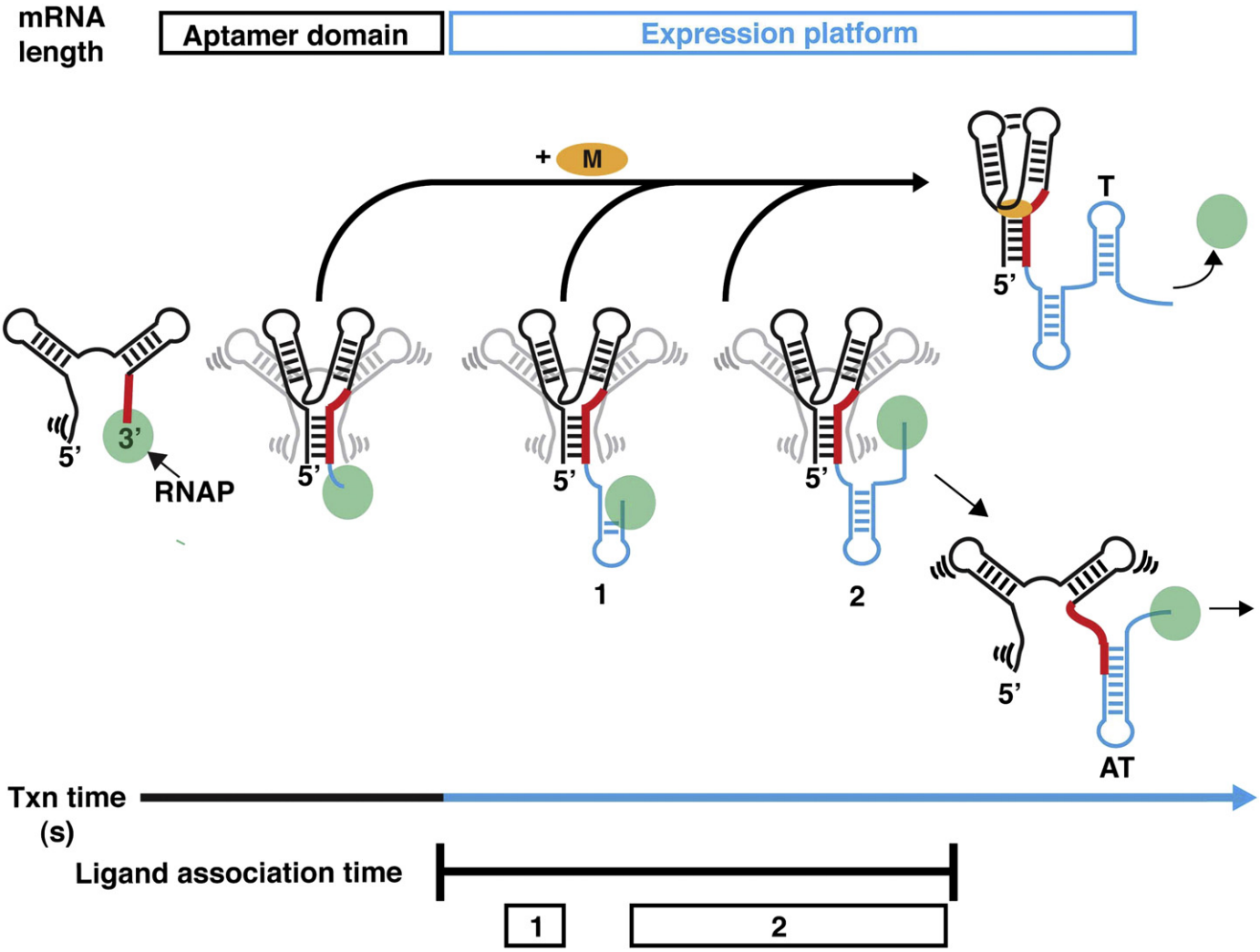

Fig. 3. A riboswitch timeline. During the aptamer time regime (black), the sensor domain is synthesized and acquires secondary structure rapidly followed by slower formation of Mg^{2+}-dependent tertiary structure. Upon formation of a competent aptamer domain the opportunity for ligand binding becomes possible. During the expression time regime (blue) the expression platform is synthesized, but the aptamer domain remains binding competent until the riboswitch has committed to forming the antiterminator element ("ligand association time"). During this time there are often two programmed transcriptional pauses (boxes 1 and 2) that stall the polymerase, and thereby influence the amount of time the riboswitch has for sensing its environment before committing to formation of one of the two mutually exclusive secondary structures in the expression platform.

potential secondary structural rearrangement in the absence of ligand. These events are slated to occur in the time required to transcribe the full riboswitch (the "expression" time regime; blue, Fig. 3). As bacterial polymerases have average elongation rates of ~50 nucleotides per second [59], it appears that riboswitches must have strict kinetic constraints imposed upon them, though the degree to which this holds true may vary depending on the overall length and sequence composition of the expression platform of a given transcript.

To study the kinetics of folding during the "aptamer" time regime of an adenine binding riboswitch, a recent study by Block and coworkers applied single molecule force spectroscopy [60]. The rate of RNA refolding was monitored by fully extending the RNA under high force, followed by measuring the probability of obtaining fully refolded RNA as a function of time. This was done in the presence of adenine concentrations ranging from 1 to 200 μM to obtain true rate constants from the pseudo-first order constants determined at each concentration. This analysis yielded a rate constant of 0.4 s^{-1} for formation of a competent receptor, and k_{on} and k_{off} of values of 8×10^4 M^{-1} s^{-1} and 0.2 s^{-1} respectively for the ligand binding step. This translates to time constants (time required for ~37% of the reaction to go to completion) of approximately 3 s for folding, 4 s for ligand association (assuming a concentration of 3 μM adenine), and ~8 s for ligand dissociation. Thus, folding and ligand binding of the RNA aptamer occur on a timescale similar to transcription of the riboswitch (~4 s for transcription of 200 nucleotides without pausing; *vide infra*), supporting the hypothesis that the individual rate constants of folding and binding have a stronger influence on the outcome of gene expression than the equilibrium binding constant. It can also be reasoned that mutations that influence the rate of tertiary structure acquisition during the aptamer time regime (Fig. 3; blue) would prevent the receptor from forming on a timescale relevant to transcription, which has been proposed as a possible mechanism of some naturally occurring mutations that disrupt regulation by the L-box but have relatively subtle effects on equilibrium lysine binding [29,52].

A consequence of riboswitches being under "kinetic control" is that there are large differences between the affinity (the apparent equilibrium dissociation constant, K_D) of isolated aptamer domains for their effector, and the concentration of ligand required to achieve half maximal regulatory response (the T_{50}) in single round *in vitro* transcription assays. This phenomenon that has been observed for many riboswitch classes, and relates to the time required to transcribe the expression platform [10,61,62]. The most comprehensive study of the relationship of riboswitch function to the rate of transcription was conducted using the FMN riboswitch from the *B. subtilis ribD* operon [63]. The apparent K_D of the FMN aptamer domain is 10 nM while the

T_{50} is 500 nM in the absence of factors that promote transcriptional pausing [62]. However, slowing the speed of transcription by either reducing NTP concentrations, or by adding NusA (a protein that increases RNA polymerase (RNAP) pausing [64]), causes a ~2-fold decrease in the T_{50} to 200 nM. The speed of transcription and the efficiency of riboswitch regulation are therefore intimately coupled [62], underscoring the need to understand ligand recognition not in the context of isolated aptamers, but also as a part of transcription.

The induced fit binding mechanism of riboswitches, a feature shared by many RNA binding interactions, plays an important role in the regulatory mechanism of these RNAs [65,66]. As alluded to above, ligand binding in the purine riboswitch is ~10^4 slower than diffusion limited processes. This is also true of the TPP and FMN riboswitches, implying that this is a general phenomenon in riboswitches [58,60,62,63,67]. These rates suggest that effector binding to these riboswitches is far too slow to be able to control transcription at concentrations close to the equilibrium dissociation constant. In part, this explains the higher ligand concentrations needed to effect half maximal transcriptional control. However, the *ribD* FMN riboswitch revealed the importance of intrinsic RNAP pausing at uridine-rich tracts encoded in the expression platform [63]. Two pauses were identified that occur immediately after the aptamer domain and after the antiterminator (Fig. 3), giving the RNA additional time (~1 and ~10 s respectively for the *ribD* FMN riboswitch) to interrogate the cellular environment before the conformational switch can happen. Despite this pausing phenomenon, the binding reaction still does not have ample time to reach equilibrium, though it does reduce the ligand concentration needed to control transcription.

It has been proposed that programmed pausing is a general strategy employed by non-coding RNA to efficiently fold during transcription. Ubiquitous housekeeping RNAs such as RNase P, SRP RNA, and tmRNA have adapted to the temporal pressures of transcription by forming "labile" intermediates during programmed pauses in the early stages of transcription [68]. These intermediates function to sequester 5′ sequences that form long-range helices (separated by >50 nt) in the native structure, and disruption of pausing that results in loss of intermediate formation leads to less efficient folding. A reasonable speculation is that the labile intermediates can be rapidly rearranged to their native state, thus providing a mechanism for preventing the RNA from adopting stabile but inactive folds. This is analogous to riboswitches whose long-range P1 helix, which defines the 5′ and 3′ boundaries of the aptamer domain, is generally implicated in structural switching. This element would be expected to undergo rearrangement if the aptamer domain remains unbound, similar to the labile folding intermediates identified in the above RNAs.

5. Riboswitches in the cellular milieu

While structural and biochemical characterization of riboswitch–ligand interactions have illuminated many aspects of their regulatory mechanisms, these data cannot always be completely reconciled with their biological activity. This is exemplified in a recent study of the variability in the ligand responsiveness of 11 different SAM responsive transcriptional units in *B. subtilis* genome [69]. These riboswitches were demonstrated to have a ~250 fold range in K_D and T_{50} *in vitro*. Additionally, *in vivo* Northern blotting, qRT-PCR, and *lacZ* reporter analyses revealed a large degree of variability in the fold change of terminated transcription in the presence and absence of methionine: this ranged from a 1.2-fold increase in termination for the *metK* gene in the presence of methionine, to a 340-fold increase for the *metE* gene as measured by qRT-PCR. The function of the gene products is thought to be tied to this disparity. Genes involved directly in methionine biosynthesis experienced the tightest regulation, the greatest level of induction, and the longest delays before induction could be detected. Meanwhile riboswitches controlling methionine transport and genes of unknown function displayed lower levels of repression during growth in methionine supplemented media, and lower magnitudes of induction during methionine starvation. This study suggests that there are structural differences in the aptamer domain or expression platform of these SAM riboswitches may account for these disparities, motivating the need to correlate these observations to available structural and sequence based data. Furthermore, differences in the time of transcription (i.e. transcriptional pausing) at these different genetic loci may tune the response, requiring further characterization of this phenomenon for each transcript.

Interestingly, not all of these riboswitches behaved as expected. The *cysH* gene does not experience induction upon methionine starvation [69], which may be explained by previous reports that this operon is regulated primarily at the DNA level by a transcriptional repressor protein that responds to levels of the cysteine precursor *O*-acetyl-L-serine [70]. The *metK* gene on the other hand experiences a transient rise in read through product at 0.5 h, but falls back to basal levels after 1 h [69]. This expression pattern suggests that this transcript may also be regulated at either the translational level or by the effects of mRNA degradation. A consequence of overlapping post-transcriptional regulation may therefore lead to differences in riboswitch response efficiency, further highlighting the need to correlate biochemical data with biological activity.

Another motivation for studying ligand interactions in their cellular context is to determine the potential of these RNAs as novel antimicrobial targets. Ribosomal RNA sets a precedence for RNA based strategies, as its known to be the target of aminoglycoside antibiotics, and many riboswitches have already been linked to antibiotic effects of naturally occurring compounds. For example, the L-box has long been recognized as a mutational hotspot in microbes resistant to the lysine analogue *S*-(2-aminoethyl)-L-cysteine [71,72]. A recent study implicated role of the L-box in mediating the toxicity of this and other lysine analogues in *B. subtilis* [52]. However, shortly after this study was published, it was discovered that the primary target of these compounds in *E. coli* was one of the two lysyl-tRNA synthetase variants (LysRS) in this organism and the toxic effects are due to incorporation into nascent polypeptides during translation [73]. Mutations to the L-box cause inefficient repression of lysine biosynthesis genes, thus allowing lysine pools to become elevated and effectively compete for the charging of LysRS [73]. A similar study demonstrated that the TPP riboswitch serves as a mutational hotspot for relieving the toxic effects of the TPP analogue pyrithiamine pyrophosphate (PTPP) in *B. subtilis*, though many of the PTPP resistant *E. coli* mutants that were sequenced apparently acquired resistance by some other mechanism [74]. However, the toxic effects of roseoflavin, an FMN analogue, to *B. subtilis* are at least in part due to direct targeting of the riboswitch upstream of the *ribD* operon by the antimicrobial [75]. The homologous operon in *Streptomyces davawensis*—an organism that naturally produces this antibacterial agent—is also responsive to roseoflavin leaving the mechanism of this bacteria's natural resistance to this antimicrobial compound unclear, highlighting the need to understand the biological roles of riboswitches in their cellular context.

6. Conclusions

With a wealth of structural information available (currently, at least 10 individual aptamer–ligand complexes have been solved by X-ray crystallography), a significant challenge remains to correlate these data with *in vitro* and *in vivo* studies that take into account the temporal aspects of riboswitch synthesis and function. In particular, new approaches need to be developed that can readily monitor cotranscriptional riboswitch folding with an eye towards its relationship to rates of ligand binding, the role of transcriptional pausing in the expression platform, and secondary structural rearrangements and their relationship to efficient genetic regulation. Determination of,

response kinetics and regulatory capacity (i.e. fold induction or repression) *in vivo* may also reveal factors that overlap with riboswitch regulation to further tune expression; concepts that will need to be addressed to determine efficacy of targeting riboswitches for therapeutic purposes. An overarching goal of these studies will be to provide a clear link between structural and functional studies that takes into consideration the complicated folding process of a riboswitch in the cell.

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