Transcriptional and translational S-box riboswitches differ in ligand-binding properties

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ABSTRACT

There are a number of riboswitches that utilize the same ligand-binding domain to regulate either transcription or translation. S-box (SAM-I) riboswitches, including the riboswitch present in the Bacillus subtilis metI gene, which encodes cystathionine γ-synthase, regulate the expression of genes involved in methionine metabolism in response to SAM, primarily at the level of transcriptional attenuation. A rarer class of S-box riboswitches is predicted to regulate translation initiation. Here, we identified and characterized a translational S-box riboswitch in the metI gene from Desulfurispirillum indicum. The regulatory mechanisms of riboswitches are influenced by the kinetics of ligand interaction. The half-life of the translational D. indicum metI RNA–SAM complex is significantly shorter than that of the transcriptional B. subtilis metI RNA. This finding suggests that unlike the transcriptional RNA, the translational metI riboswitch can make multiple reversible regulatory decisions. Comparison of both RNAs revealed that the second internal loop of helix P3 in the transcriptional RNA usually contains an A residue, whereas the translational RNA contains a C residue that is conserved in other S-box RNAs that are predicted to regulate translation. Mutational analysis indicated that the presence of an A or C residue correlates with RNA–SAM complex stability. These analyses indicate that the internal loop sequence critically determines the stability of the RNA–SAM complex by influencing the flexibility of residues involved in SAM binding and thereby affects the molecular mechanism of riboswitch function.

INTRODUCTION

Riboswitches are cis-acting RNAs that regulate gene expression in response to specific effector molecules (1, 2). Each riboswitch class contains an aptamer domain that recognizes its cognate ligand, which include vitamins, amino acids, cofactors, nucleotides, metal ions and tRNAs (2–4). In most riboswitches, binding of the ligand to the aptamer affects the structure of an expression platform, which regulates expression of the downstream genes.

As a general rule, in an ON switch, binding of the ligand promotes expression of the regulated genes, while in an OFF switch, binding of the ligand leads to reduced expression of the regulated genes. In most ON switches, the effector molecule is utilized either as a substrate or exported out of the cell, resulting in a "feed-forward" mechanism. In contrast, in most OFF switches, the associated genes are involved in biosynthesis or uptake of the effector molecule, resulting in a negative feedback control. The diversity of riboswitch-regulated genes and their recognized ligands leads to a number of exceptions to these general principles.

Riboswitches are found primarily in bacteria, where they regulate gene expression at the level of transcription attenuation or translation initiation. Transcriptional riboswitches generally contain an intrinsic transcriptional terminator that leads to premature transcription termination. Folding of the RNA into an alternate antiterminator structure allows transcription to continue and the gene is expressed. In an OFF switch, the antiterminator is more thermodynamically stable, and binding of the ligand causes stabilization of the terminator helix to repress gene expression. Translational riboswitches operate similarly, except that the terminator helix is replaced by a sequestator helix that occludes the Shine-Dalgarno (SD) region and prevents translation of the downstream gene, and the antiterminator is replaced by an antisequestrator helix that allows the ribosome to bind to the RNA and initiate translation (2).

The simplest model for riboswitch-mediated regulation suggests that the RNA senses physiological levels of the ligand and the resulting change in RNA structure triggers the regulatory activity. However, for some transcriptional riboswitches, a higher ligand concentration is required to direct gene regulation than is expected based on the apparent equilibrium dissociation constant (K_D) (5–7). It was proposed that for transcriptional riboswitches, both the kinetics of
ligand binding and the rate of transcription play an important role in the regulatory decision (6, 8). During transcription, the aptamer domain is transcribed and folded first, with only a short window of opportunity for the ligand to bind before RNA polymerase (RNAP) reaches the decision point between termination and antitermination. For an OFF switch, if the aptamer domain folds and binds the ligand before RNAP completes transcription of the antiterminator sequence, the competing terminator helix forms and termination occurs. However, if the ligand does not bind to the RNA in this timeframe, the more stable antiterminator forms and transcription continues. Due to the speed of transcription, most transcriptional riboswitch RNAs do not reach equilibrium with the ligand before RNAP reaches the decision point (8).

Occupancy is also affected by the rate of dissociation of the ligand from the RNA (9, 10). Translational riboswitches differ from their transcriptional counterparts in that they can bind the ligand both co-transcriptionally and post-transcriptionally. They therefore have the potential to establish thermodynamic equilibrium with their ligand (9, 11). If the half-life of the RNA-ligand complex is shorter than the half-life of the fully transcribed mRNA, a translational riboswitch has the potential to make multiple reversible regulatory decisions in its lifetime.

The S-adenosylmethionine (SAM)-responsive S-box (SAM-I) RNAs are one of the most prevalent classes of riboswitches and are predominantly found in Firmicutes (3). S-box riboswitches regulate genes that are involved in biosynthesis and uptake of methionine and SAM, and operate primarily at the level of transcription attenuation (12). The well-characterized Bacillus subtilis yitJ transcriptional S-box riboswitch forms a highly stable complex with SAM in vitro, with a half-life of 7.2 min (7). The high stability of the complex indicates that once bound, SAM is not likely to dissociate in the time scale of transcription, making the binding and regulatory decision irreversible (13). The Smk-box (SAM-III) RNAs, another class of SAM-sensing riboswitches that regulate SAM synthetase (metK) genes at the level of translation initiation, exhibit a much less stable RNA-SAM complex, with a half-life of ~8 s (9). A long Smk-box transcript half-life (~3 min) provides the opportunity for multiple rounds of SAM association and dissociation, suggesting that the translational Smk-box riboswitch can act as a true reversible riboswitch and make multiple regulatory decisions in its lifetime (9, 14, 15).

The SAM-responsive transcriptional S-box and translational Smk-box riboswitches employ different regulatory strategies (irreversible vs. reversible) to regulate their respective genes. However, the two riboswitches are structurally different and recognize SAM using different mechanisms. Hence, it is unclear whether it is the level of regulation or the riboswitch structure that dictates the differences in the regulatory mechanisms. We therefore chose to compare the properties of transcriptional and translational SAM-responsive riboswitches from the same class in different organisms. We previously showed that different B. subtilis S-box genes show different properties (7); we therefore compared properties of the same gene.

Our data show that the stability of the translational S-box RNA-SAM complex is significantly (8.6-fold) lower than the stability of the transcriptional S-box RNA-SAM complex, which may enable the RNA to make more than one reversible regulatory decision, unlike its transcriptional counterpart. We investigated elements within the riboswitch that might contribute to the differences in SAM binding properties and identified a nucleotide in the second internal loop of helix P3, the identity of which correlates with predicted transcriptional vs. translational regulation. Mutational analysis indicated that the identity of this residue affects the stability of the RNA-SAM complex, and selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) analysis provided insight into the structural basis for the differences in the ligand binding properties. These results indicate that this internal loop sequence plays an important role in determining the stability of the RNA-SAM complex in both transcriptional and translational RNAs, and therefore affects the molecular mechanism of S-box riboswitch function.

RESULTS
Identification of a translational S-box riboswitch

Bioinformatic analyses have suggested that in some non-Firmicutes, S-box riboswitches regulate gene expression by mechanisms other than transcription attenuation, based on the absence of a distinct terminator helix in the expression platform (16–18). We used the Rfam database (19) to identify S-box sequences that were likely to regulate gene expression at the level of translation initiation, and for which the riboswitch of the equivalent gene has been characterized in B. subtilis.

The metL S-box riboswitch from Desulfurispirillum indicum, a member of the phylum Chrysiogetes (20), has all of the conserved features (18, 21) of the S-box RNA family (Figure 1A). The expression platform of this RNA is predicted to fold into two competing structures, a sequestrator helix that occludes the putative SD sequence and an antisiequestrator helix that exposes the SD region. The metL gene encodes a putative cystathionine γ-synthase, which catalyzes the conversion of cysteine to cystathionine. This gene is followed by the gene encoding cystathionine β-lyase, which catalyzes the conversion of cystathionine to homocysteine. In B. subtilis, these enzymes are encoded by the metLC operon (7), which is regulated by a transcriptional S-box riboswitch (Figure 1B).

Transcriptional and translational metL RNAs bind to SAM with similar affinity and specificity

The aptamer domain of the D. indicum metL RNA contains the elements that have been shown previously to be important for SAM binding and for formation of structural elements such as the kink-turn and the pseudoknot (13, 22). We therefore compared the SAM binding properties of the D. indicum metL aptamer RNA to those of B. subtilis metL and other previously characterized S-box riboswitches (7).

Size-exclusion filtration assays were performed to determine the SAM binding affinities of the two metL riboswitches. The B. subtilis glyQS RNA, which is a T-box riboswitch that binds tRNA\textsubscript{Gly} instead of SAM, was used as a negative control. The D. indicum metL aptamer RNA bound SAM with an affinity comparable to that of the B. subtilis metL RNA (Figure 2A). A G residue in the junction between helices P1 and P2 (J1/2) was previously shown to interact directly with SAM, and mutation of that G to C causes loss of SAM binding in the yitL S-box aptamer RNA (13). Analogous mutations (G19C for D. indicum metL RNA, G39C for B. subtilis metL RNA) resulted in undetectable levels of SAM binding (Figure 2B), consistent with previous results for yitL. S-box riboswitches have also been shown to strongly discriminate against S-adenosylhomocysteine (SAH), which is a natural analog of SAM (12). To test whether the D. indicum S-box RNA exhibits similar selectivity against SAH, competition binding assays were performed with \textsuperscript{3}H-SAM, unlabeled SAM and SAH. We found that unlabeled SAM but not SAH competed efficiently with \textsuperscript{3}H-SAM for binding to the aptamer RNAs, which indicates that the binding is specific for SAM (Figure 2B). These data show that the aptamer domains of both transcriptional and translational metL S-box riboswitches exhibit affinity and specificity for SAM, as well as sensitivity to a mutation in J1/2, similar to that of the previously characterized S-box riboswitches (7, 12, 13).

The D. indicum metL riboswitch undergoes a SAM-dependent structural transition and binds 30S ribosomal subunits in vitro

The translational regulation model suggests that the sequestrator helix in the D. indicum metL RNA undergoes a SAM-dependent structural transition that results in stabilization of the sequestrator helix that contains the SD region. We performed an RNase H cleavage assay to determine if the accessibility of the SD region decreases in response to SAM, as predicted by the model. The RNA was incubated with a DNA oligonucleotide complementary to the SD region in the presence or absence of SAM and the availability of the SD region was monitored by addition of RNase H, which cleaves RNA-DNA hybrids. A cleavage product was observed only in reactions that contained the oligonucleotide but lacked SAM (Figure 3A), consistent with SAM-dependent stabilization of the sequestrator helix. These results indicate that the SD sequence is accessible to oligonucleotide binding (and therefore RNase H cleavage) in the absence but not in presence of SAM, supporting the translational regulation model.
SAM-dependent stabilization of the sequestrator helix is predicted to inhibit binding of the ribosome to the SD region, whereas in the absence of SAM, the formation of the antisequestrator helix should make the SD sequence available for ribosome binding. To test this hypothesis in vitro, we performed a primer extension inhibition (“toeprint”) assay. In this assay, when a transcribing reverse transcriptase (RT) encounters an obstacle on the RNA (e.g., the 30S ribosomal subunit or a strong secondary structure), it stalls, and primer extension is inhibited. When the initiator tRNA$^{\text{Met}}$ is positioned in the P site of a bound 30S subunit, an RT stop (toeprint) is usually observed 16-17 nt downstream of the A of the AUG start codon (23, 24). D. indicum metI RNA was incubated with E. coli 30S ribosomal subunits and initiator tRNA$^{\text{Met}}$ in the presence or absence of SAM. Strong RT stops were observed at positions +16 and +17 nt downstream of the A in the AUG start codon in the absence of SAM. The intensity of the toeprint signal decreased ~2-fold (2.1 ± 0.2; SD, n = 3) in the presence of SAM (Figure 3B). This suggests that addition of SAM shifts the RNA population into a conformation that inhibits ribosome binding, supporting the hypothesis that the SAM binding represses translation initiation. In the presence of SAM (Figure S1, lane 2), RT stops were also observed at the 3’ ends of the aptamer domain (G125, C127) and the sequestrator helix (G188, A189). This suggests that both the structures are stabilized in the presence of SAM, which supports our model of the OFF conformation. Interestingly, the intensity of these stops decreased upon addition of the 30S subunit and tRNA$^{\text{Met}}$ (Figure S1, lane 5), consistent with the model that the binding of 30S subunit can stabilize the ON conformation.

Stability of the transcriptional and translational RNA-SAM complexes in vitro

Although the SAM-binding affinities of the transcriptional and translational RNAs were similar, the kinetics of SAM interaction may play an important role in dictating the regulatory strategies (25–27). The kinetics of ligand dissociation determines the stability of the RNA-ligand complex. A less stable RNA-ligand complex may provide an opportunity for multiple rounds of regulation by the riboswitch while a more stable RNA-ligand complex could make the regulatory decision irreversible.

We determined the stability of the transcriptional metI S-box RNA-SAM complex using a 2-aminopurine (2AP)-based fluorescence assay. 2AP fluorescence is sensitive to local conformational changes in RNA (28), such that the fluorescence intensity decreases in response to base stacking and ligand binding interactions. A14 of helix P1, which is a part of the SAM-binding core (Figure 4A), was replaced with 2AP. This residue stacks with U15 and base pairs with U121, both of which specifically recognize the sulfonium ion in SAM (13, 29). SHAPE-based structural analyses have shown that the A at this position undergoes a SAM-dependent decrease in reactivity (13, 30). 2AP at position A14 is predicted to fluoresce in the absence of SAM and exhibit quenching in the presence of SAM. Previous work from our lab showed that a 2AP substitution in the same position in the yitJ aptamer domain resulted in SAM-dependent quenching of fluorescence (31). A bipartite RNA consisting of two pieces of RNA (a chemically synthesized 5’ half with 2AP at the A14 position and a 3’ half synthesized by T7 RNAP transcription) that were co-folded to reconstitute the aptamer domain was used to assess SAM binding to the translational metI S-box RNA. The bipartite RNA bound SAM with an affinity ($K_d = 99$ nM) that was within two-fold of that of the intact RNA ($K_d = 53$ nM). A similar bipartite system was used to study the kinetics of SAM dissociation from the SmK-box riboswitch (9). To determine the rate of SAM dissociation, the equilibrated bipartite metI RNA-SAM complex was mixed rapidly with a 10-fold molar excess of competitor B. subtilis yitJ S-box RNA. The yitJ RNA binds SAM with a high affinity ($K_d = 19$ nM) and forms a very stable complex with a half-life of 7.2 min (7). As the SAM dissociates from the metI RNA-SAM complex, it is sequestered by the yitJ RNA and the fraction of SAM-bound RNA decreases. This leads to an increase in the 2AP fluorescence emission over time and the data are plotted as the fraction of SAM-bound RNA as a function of time.

The data were initially fit with a single exponential equation but improvement in the fit was observed by using a double exponential
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The fast and slow dissociation rates \( (k_{off}) \) were determined to be 0.23 s\(^{-1}\) and 0.014 s\(^{-1}\), respectively (Figure 4C). The observed fast rate is likely to be due to a dynamic intermediate state that might exist between the bound and unbound states of the S-box aptamer. The \( t_{1/2} \) value, which indicates the stability of the RNA-ligand complex, was determined from the slow rate as 50 ± 2 s. The association rate \( (k_{on}) \) derived from the \( K_D \) and the \( k_{off} \) values is \( 2.6 \times 10^5 \) M\(^{-1}\)s\(^{-1}\). The time required for the ligand to bind the RNA will depend on the physiological concentration of SAM in \( D. indicum \). For comparison, the half-life of the translational \( E. faecalis \) \( S_{MK} \)-box RNA in vivo was determined to be \( \sim3-4 \) min (9). The average half-life of mRNAs in a gram-negative non-Firmicute like \( E. coli \) is \( \sim5-7 \) min (32, 33). Assuming a half-life value in a similar range for the \( metI \) transcript, the \( t_{1/2} \) value suggests that SAM can bind and dissociate more than once before the \( metI \) mRNA is degraded. The ability for multiple rounds of SAM association and dissociation implies that the riboswitch can make multiple reversible regulatory decisions in its lifetime.

Next, we determined the stability of the transcriptional \( metI \) RNA-SAM complex (Figure 4D). We could not derive an off-rate from a similar 2AP fluorescence-based stopped-flow assay, as SAM failed to dissociate in the timescale of the experiment. We therefore used the size exclusion filtration-based assay with radioactive SAM as ligand and non-radioactive SAM as the competitor ligand. In this assay, the preformed RNA-radioactive SAM complex was mixed with excess cold SAM and aliquots were removed at various time points and passed through the filter to separate RNA-bound SAM and unbound SAM. The amount of radioactive SAM bound to RNA was determined for each aliquot and data were plotted as the fraction of SAM bound to RNA as a function of time. The \( t_{1/2} \) value calculated from the plot was \( 7.2 \pm 0.2 \) min (Figure 4D), which is similar to the previously reported value of 5.1 min (7). The \( k_{off} \) value is 0.0016 s\(^{-1}\) and the \( k_{on} \) value is \( 3.5 \times 10^4 \) M\(^{-1}\)s\(^{-1}\). Since the rate of prokaryotic transcription is estimated to be \( \sim25-30 \) nt s\(^{-1}\) (34–36), a complex half-life in minutes suggests that both binding and regulatory decision would be irreversible in the time frame of transcription.

The translational \( metI \) riboswitch-SAM complex is 8.6-fold less stable than the transcriptional \( metI \) riboswitch-SAM complex, despite similarity in the overall secondary structure, core binding sequences and binding affinities. The low stability suggests that it can reach thermodynamic equilibrium with its ligand. Hence, the transcribed mRNA is predicted to monitor and respond to changes in the physiological levels of SAM that are closer to the \( K_D \). In contrast, the high stability for the transcriptional \( metI \) riboswitch-SAM complex suggests that it will likely never reach equilibrium with SAM. Consequently, a concentration of SAM higher than the \( K_D \) will be required to trigger the termination response. These data suggest that the translational and transcriptional S-box riboswitches can adopt different regulatory strategies to regulate the same gene in different organisms.

Role of helix P3 in RNA-SAM complex stability

The overall secondary structure and identity of the nucleotides in the SAM-binding core are conserved among S-box sequences, whereas elements outside of the binding core show variability (7, 21, 37, 38). For example, while the residues at the proximal end of helix P3 that form the first internal loop (IL3a) are highly conserved and directly contact SAM (13, 29), the distal end of helix P3 varies in both length and sequence. Helix P3 contains a second internal loop (IL3b), the size and nucleotide composition of which are variable (39). It was previously shown that deletion of IL3b is detrimental to the function of the \( B. subtilis \) \( metE \) S-box riboswitch, and the presence of an A in the 3' side of IL3b promoted SAM binding and riboswitch activity (39). The adenine corresponding to A104 in the 3' side of IL3b in \( B. subtilis \) \( metI \) riboswitch (Figure 4B, in red) is highly conserved and is present in > 75% of S-box sequences (19).

We compared the sequence of IL3b between the transcriptional and translational RNAs. Whereas the 3' side of IL3b of the transcriptional \( metI \) RNA contains the more common 2-nt bulge including the conserved A (A104, Figure 4B), the translational \( metI \) RNA lacks this conserved A and
contained only a single cytosine residue (C84, Figure 4A) at the same position. Analysis of the Rfam seed sequence alignment showed that a single C in this position is found in <1% of S-box sequences, all of which are predicted to regulate translationally based on the lack of a predicted transcriptional terminator and the presence of putative sequestrator structures. We hypothesized that the difference in the composition between the second internal loops of the two RNAs could contribute to the difference in their RNA-SAM complex stabilities.

To test this hypothesis, we first replaced C84 in the translational metl RNA with two adenine residues (C84A/ΩA85), to mimic IL3b of the transcriptional metl RNA. This mutant has an RNA-SAM complex half-life of 280 ± 10 s (Figure 4C), a 5.6-fold increase. Replacing A104 and A105 in the transcriptional RNA with a single C (A104C/ΔA105) resulted in an RNA-SAM complex half-life of 2.0 ± 0.4 min (Figure 4D), a 3.6-fold decrease in complex stability. These results support our hypothesis that the difference in the kinetics of ligand dissociation between the transcriptional and translational RNAs is, at least in part, influenced by the difference in the size and nucleotide composition of the IL3b region.

**SHAPE analysis of transcriptional and translational RNAs**

To investigate the structural basis for the difference in the stabilities of the RNA-SAM complexes between the two RNAs, we examined the structure of the wild type and mutant translational and transcriptional metl RNAs using selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) (40). SHAPE measures the backbone flexibility of each nucleotide in an RNA, such that unconstrained nucleotides are preferentially acylated and show higher reactivity relative to constrained nucleotides. Comparison of the SHAPE reactivity profiles between the wild-type transcriptional and translational RNAs showed significant differences in the flexibility of the conserved A residues in the 5’ side of IL3a. The nucleotide identity of the position corresponding to the first A (translational, A54; transcriptional, A74) is 97% conserved (19, 38), while that corresponding to the second A (translational, A55; transcriptional, A75) is 96% conserved (39). In the

*B. subtilis* *yitJ* S-box RNA crystal structure with SAM, the first A in IL3a is a part of the conserved A●U sheared base pair (translational, A54●U87; transcriptional, A74●U108) that directly contacts SAM by forming a base triple with its adenosyl moiety (13, 29). The second A in IL3a (A75 in Figure 4B) interacts with the conserved A in IL3b (A104 in Figure 4B, marked in red).

In the translational metl RNA, both the A residues in IL3a (A54 and A55; green nucleotides in Figure 4A) were significantly more reactive (Figure 5A) than the corresponding A residues (A74 and A75; green nucleotides in Figure 4B) in the transcriptional metl RNA (Figure 5C). Introduction of mutations (C84A/ΩA85) in IL3b of the translational metl RNA led to a 2-fold and 4.5-fold decrease in the reactivities of A54 and A55, respectively (Figure 5B). In contrast, in the transcriptional metl RNA, A74 in IL3a was reactive while A75 was minimally reactive (Figure 5C). The mutations (A104C/ΔA105) in IL3b of the transcriptional metl RNA led to a 3.4-fold increase in the reactivity of A75 (Figure 5D). In both cases, the second A in IL3a exhibited a larger change in the reactivity in response to the mutations.

These results indicate that the presence of A residues in the 3’ side of IL3b decreases flexibility of the A residues (shown in green in Figures 4A and 4B) in the 5’ side of IL3a. In contrast, the presence of C at the same position increases flexibility of the A residues in the 5’ side of IL3a. The change is most prominent in the second A residue of IL3a, which contacts the 3’ side of IL3b. This suggests that the nucleotide identity and composition of IL3b may influence the dynamics of residues in IL3a. Since the residues in IL3a directly contact SAM, the change in local flexibility of these residues could affect the kinetics of ligand interaction. These data suggest that the residues in IL3b, through their interaction with residues in IL3a, could indirectly affect the stability of the RNA-SAM complex. The composition of IL3b may therefore play a role in determining the regulatory mechanisms employed by transcriptional vs. translational S-box riboswitches.

**DISCUSSION**

Few studies have directly compared transcriptional and translational riboswitches for a specific
riboswitch class. For example, transcriptional and translational preQ1 riboswitches fold via two distinct pathways of conformational selection and induced fit mechanisms, respectively, despite their structural similarity (41). Comparison between the adenine-binding translational add and transcriptional pbaE riboswitches suggested that the level of gene regulation might be important in determining the riboswitch regulatory strategies (42). It was shown that the add riboswitch can switch reversibly between the ON and OFF states but pbaE RNA could not (42–44). These riboswitches regulate genes that are functionally distinct (adenosine deaminase vs. an adenine efflux pump), so it is unclear if their regulatory mechanisms correlate with gene function. More importantly, the role of RNA-ligand complex stability in the regulatory strategies of transcriptional vs. translational riboswitches was not investigated.

The rate of association is ~10 times slower for the transcriptional RNA when compared to the translational RNA. The $k_{on}$ value of 3.5 x $10^4$ M$^{-1}$s$^{-1}$ for the B. subtilis transcriptional riboswitch suggests that in the presence of 1 µM SAM, it would take ~20 s for half of the metI transcripts to bind SAM. Increasing the SAM concentration would decrease the association time constant proportionately. During the SAM limiting conditions (<25 µM) (7), binding will take around ~1 s, while under saturating levels of SAM (300 µM) it could take ~0.1 s. For B. subtilis metI, there are 24 nts between the end of aptamer domain and the end of the antiterminator helix. This means that after the aptamer is transcribed, it has ~1 s (assuming a transcription elongation rate of 25 nt/s) to fold and bind SAM. Riboswitch aptamers have been shown to fold in less than 100 ms (45, 46). The probability of SAM binding to the aptamer before the antiterminator helix formation is therefore higher when SAM is at saturating levels. Once the aptamer is transcribed, it will take ~1.5 s for RNAP to transcribe the stretch until terminator and the run of Us (~40 nts). The half-life of 7.2 min suggests that if SAM is bound to the aptamer RNA, it is unlikely to dissociate in this interval. Hence, both the binding and regulatory decision for the metI transcriptional riboswitch is irreversible in the timeframe of transcription. The SAM pools of D. indicum are not known. However, the ~10 times faster on and off-rates support the possibility of multiple rounds of SAM association and dissociation.

Our data indicate that the presence of a C in the 3’ side of IL3b imparts low stability to RNA-SAM complexes. A metE S-box riboswitch mutant that contains a C in IL3b exhibited loss of SAM binding and riboswitch function (39), suggesting that a C at this position can be detrimental to SAM binding in other S-box riboswitches as well. It remains to be seen how the presence of other residues in IL3b would affect the dissociation kinetics and hence the regulatory mechanism of S-box riboswitches. The presence of a single C residue in the 3’ side of IL3b appears to be associated only with the translational S-box riboswitches. Based on the low stability of the RNA-SAM complex, we predict that the translational riboswitches with this sequence will regulate their genes reversibly. However, there are some S-box sequences that we predict regulate translationally based on the lack of a terminator helix that do not have the single C residue in the 3’ side of IL3b. Characterizing RNAs of this type may provide additional insight into the role of this domain in S-box RNA function.

Like the metI transcriptional riboswitch, most of the other B. subtilis transcriptional S-box riboswitches have an A in the IL3b, and form stable complexes with SAM, with half-lives ranging from 4.3–11 min (7). One exception is yxjG, which is predicted to form a weaker pseudoknot base pairing interaction (2 nt vs 3-4 nt), which could contribute to the lower complex stability (1.6 min). The B. subtilis yoaD RNA that lacks IL3b has an RNA-SAM complex half-life of 1.8 min. These observations provide additional support for the hypothesis that the presence and composition of IL3b plays a role in the ligand dissociation kinetics. D. indicum contains a second S-box riboswitch in the metA gene that regulates at the level of transcription attenuation in vitro (Figure S3). In contrast to the translational metI riboswitch, the 3’ side of IL3b of the metA transcriptional RNA contains the highly conserved and more predominant A residue, which is associated with highly stable RNA-SAM complexes and irreversible regulation.
Interestingly, the differences in the composition of an internal loop in the P2 helix of TPP riboswitch is suggested to contribute towards differences in the regulatory mechanisms between the *E. coli thiM* and *A. thaliana thiC* TPP riboswitches (47). The nucleotides in this internal loop are either part of or adjacent to the binding pocket and important for TPP binding and folding of the riboswitch (48). Hence, the contribution of an internal loop to regulatory differences between riboswitches in the same class could be a feature found in other riboswitch families.

Residues adjacent to the ligand binding pocket have been suggested to modulate ligand binding kinetics in the purine and FMN riboswitches (10, 49). However, no correlation was observed between the variation in ligand binding kinetics and the degree of local nucleotide flexibility (10). Our SHAPE data link the kinetics of SAM dissociation to the dynamics of nucleotides adjacent to the SAM binding pocket in the S-box riboswitch. In the SAM-bound *yitJ* crystal structure (13), the residues corresponding to A74 and U108 (Figure 4B) form a base triple with the adenosyl moiety of SAM. The residue corresponding to A75 in IL3a is part of a dinucleotide platform (A75●C76-G107) that is suggested to further stabilize the position of the adenine ring of SAM (29, 50). The residues corresponding to A75 in IL3a and A104 in IL3b interact via hydrogen bonds and stacking interactions. The amino groups of A75 and A104 in IL3b interact via hydrogen bonds and the 2'-hydroxyl groups of the other. In the SAM-free *metF-H2* S-box crystal structure (51) that lacks IL3b, this interaction is missing. Instead, the A corresponding to A75 in IL3a blocks the SAM-binding pocket by forming a base triple with residues corresponding to A74 and U108.

Unlike the A75-A104 interaction in the transcriptional *metI* RNA, the interaction between C84 in IL3b and A55 in IL3a in the translational *metI* RNA could either be weak or absent. This is because A55 is 4.5-fold more flexible in the presence of C in IL3b (Figure 5A and 5B). Additionally, a ΔC84 deletion mutant exhibited an RNA-SAM complex half-life (Figure S2B; 54 s) similar to that of the wildtype translational RNA. In the absence of this interaction, the translational aptamer could behave similarly to the SAM-free *metF* crystal structure that lacks IL3b. The residue corresponding to A55 was proposed to transition rapidly between two major equilibrium conformations, one that occupies the binding pocket and another that is flipped out of the pocket (51). This means that A55 in the *metI* translational RNA could compete with SAM for the binding pocket, which could explain the faster dissociation kinetics. In contrast, the A104-A75 interaction in the *B. subtilis metI* transcriptional RNA would prevent A75 from occupying the binding pocket, making the binding pocket less dynamic and could encourage a SAM-bound state by stabilizing a binding-competent aptamer RNA.

The regulatory mechanism of the translational riboswitch is likely to be influenced by a combination of additional factors, such as the strength of the SD sequence, dynamics of expression platform, cellular concentration of the ligand, the competition between kinetics of ligand binding to the aptamer domain and ribosome recruitment to the expression platform. The variation in ligand binding properties of the 11 *B. subtilis* transcriptional S-box riboswitches have been linked to the function of the genes they regulate (7). Similarly, the reversibility of translational S_MK-box and *add* riboswitches has been speculated to be linked to their physiological roles (9, 42). It is possible that the regulatory strategy adopted by the translational *metI* S-box riboswitch provides an advantage to its host organism in its niche. *D. indicum* was isolated from arsenite rich environments and could depend on detoxification by SAM-dependent methylation of arsenite (20, 52, 53). Therefore, rapid sensing of physiological levels of SAM and maintaining SAM homeostasis may be critical for its survival. Investigation into these aspects, both in vitro and in the context of the native host, will enable a better understanding of the basis for the regulatory differences observed between transcriptional and translational S-box riboswitches.

**EXPERIMENTAL PROCEDURES**

**DNA constructs and RNA synthesis**
The sequences for transcriptional *metI* constructs were derived from the genome of *D. indicum* strain S5 (NCBI CP002432). Transcriptional S-box sequences were obtained from the genome of *B. subtilis* strain 168 (NCBI AL009126). DNA
constructs were generated using either oligonucleotide (Integrated DNA Technologies) as templates for PCR amplification. Constructs containing mutations were made by replacing a pair of oligonucleotides with an alternate pair that contained the sequence changes. All constructs were confirmed by DNA sequencing. RNA was synthesized using either laboratory prepared T7 RNAP or a TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific). All transcripts synthesized using T7 RNAP were designed to contain a G residue as the first nucleotide, to facilitate transcription initiation. The RNA products were gel-purified using denaturing polyacrylamide gel electrophoresis (PAGE). RNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

SAM-binding assays
Equilibrium dissociation constants were determined using a size-exclusion filtration assay as described previously (7). Aptamer RNA (0.1 μM) was incubated at 37°C for 30 min with varying concentration of 3H-SAM (1 mCi/ml) and passed through BSA-treated 10K Nanosep Omega filter microconcentrators (Pall). Material retained by the filter was mixed with Packard Bioscience Ultima Gold scintillation fluid and counted in a Packard Tri-Carb 2100TR liquid scintillation counter. Nonlinear regression analysis was performed using GraphPad Prism 8 software. The data were fit to the following quadratic equation: 

\[ Y = \frac{[(K_m + M_T + L_T)^2 - (K_m + M_T + L_T)^2 - 4*M_T*L_T]}{2*M_T}; \]

Here, \( M_T \) is the total RNA concentration and is constant; \( L_T \) is the ligand concentration; \( Y \) is the fraction of bound RNA. The graph is plotted as fraction bound vs. \( [L_T] \).

Competition binding assays were performed by incubating RNAs (0.1 μM) with 3H-SAM (0.1 μM) at 37°C for 30 min followed by addition of 50-fold molar excess unlabeled SAM or SAH. Samples were filtered as above, and 3H-SAM retained by the filter was counted in the liquid scintillation counter.

RNase H cleavage assay
RNase H cleavage experiments were carried out with D. indicum metI RNA uniformly labeled by transcription in the presence of [\( \alpha - ^{32}P \)]-UTP (800 Ci/mmol, Perkin Elmer). An antisense oligonucleotide (TCCCCCT) complementary to the SD region (nts 180-186) was added to SAM-bound and SAM-free RNA (40 nM) at 100-fold molar excess and the reactions were incubated at 37°C for 5 min. RNase H (10 U/μL; Ambion) was added and the reactions were incubated at 37°C for 10 min. The reactions were terminated by phenol-chloroform extraction, and products were resolved by denaturing 6% (wt/vol) PAGE and visualized by PhosphorImager analysis (Molecular Dynamics).

**Primer Extension Inhibition Assay**
A DNA primer complementary to the positions 277-302 of the 3’ end of the D. indicum metI transcript was 5’ end-labeled (54) with \([\gamma - ^{32}P ]\)-ATP (6000 Ci/mmol, Perkin Elmer). The labeled oligonucleotide (10 nM) was annealed to the full-length T7 RNAP-transcribed RNA (10 nM) corresponding to positions 7-302 of the metI RNA. SAM (160 μM) or water was added, and the samples were incubated for 10 min at 37°C. Purified E. coli 30S ribosomal subunits (1.5 μM; kindly provided by the Kurt Fredrick lab) and E. coli tRNA^Probes (0.9 μM; tRNA Probes, Texas) were added, and the complexes were incubated at 37°C for 30 min. Primer extension reactions were carried out as described previously (55). Reactions were quenched by the addition of gel-loading buffer (Ambion) and resolved by using 6% denaturing PAGE. A DNA sequencing ladder was generated by using a DNA Sequenase 2.0 Kit (USB), a DNA template corresponding to positions 7-302 of the D. indicum metI RNA, and the same downstream primer as used in the primer extension assay. Products were visualized by using PhosphorImager analysis (Molecular Dynamics) and quantified by using ImageQuant 5.2 software.

**Measurement of RNA-SAM complex stability in vitro**
The size-exclusion filtration binding assay was modified to determine the stabilities of the wild-type and mutant transcripational metI RNA-SAM complexes. Aptamer RNA (1 μM) was denatured, slow cooled, and incubated with 3H-SAM (2 μM) at 37°C for 30 min. Next, unlabeled SAM (200 μM) was added as a competitor ligand. Samples were removed at intervals and were passed through BSA-treated Nanosep 10K filters. The amount of 3H-SAM retained by the filter was counted in a liquid
scintillation counter. Complex half-life was determined by nonlinear regression analysis using GraphPad Prism 8 software.

The stabilities of the wild-type and mutant translational metI RNA-SAM complexes were measured using stopped-flow spectroscopy with a bipartite RNA as described previously (9). The upstream RNA, corresponding to positions 7-60 of the D. indicum metI leader RNA, contained a 2AP substitution at position A14 purchased from Dharmacon. The downstream RNA corresponded to positions 61-128 of the D. indicum metI leader RNA and was generated by T7 RNAP transcription. The first base pair of this half of RNA was changed from C-G to G-C to introduce G61 as the initiating nucleotide to facilitate transcription. The RNA segments (2 µM each) were co-folded in 1X buffer (20 mM Tris HCl [pH 8.0], 20 mM NaCl, 10 mM MgCl2, 0.1 mM EDTA) by heating the mix at 65°C for 5 min and slow cooling to 40°C. SAM (2 µM) was added after slow cooling and reactions were incubated at 37°C for 30 min to allow the complex to reach equilibrium. A competitor RNA (10 or 20 µM) corresponding to positions 14–151 relative to the transcription start site of the B. subtilis yitJ leader RNA was folded separately using similar folding conditions. Kinetic experiments were performed in an SX20-MV spectrometer (Applied Photophysics) using an excitation wavelength of 310 nm. Equal volumes of preformed metI S-box RNA–SAM complex and competitor yitJ leader RNA were rapidly mixed and fluorescence emission was monitored after passage through a 360 nm cut-off filter. The raw trace (Figure S2A) represents an increase in the fluorescence over time as the 2AP fluorescence unquenches due to SAM dissociation. The fraction of RNA bound to SAM was set to 1 at T0 and the data were plotted as fraction bound vs. time. The reported rate is the mean of three independent experiments. For each experiment, at least 5 individual traces were measured. The data from these experiments were averaged and fit to either a single or double exponential equation using GraphPad Prism 8 software to determine the dissociation rate constants (k_off). The complex half-lives were determined from the off rate as t_{1/2} = ln (2)/k_{off}. The k_on value was determined from the equation K_D = k_{off}/k_{on}. For the mutant translational RNA, the data were collected for 1000 s and the reported half-life value was derived from this plot.

SHAPE

D. indicum metI aptamer RNA (nts 7-128) and B. subtilis metI RNA (nts 28-150) were introduced into the SHAPE cassette (40). The RNAs (0.4 µM) were folded by heating and snap cooling in a modified SHAPE buffer (100 mM HEPES [pH 8.0], 10 mM MgCl2, 20 mM NaCl). The RNAs were modified with N-methylisatoic anhydride (NMIA, 3.25 mM; Invitrogen) and precipitated as described (40). Primer extension inhibition and RNA sequencing reactions were carried out in parallel using a fluorescently labeled primer (5’-GAACCGGACCGAAAGCCC-3’; Applied Biosystems, ThermoFisher Scientific) that is complementary to the SHAPE cassette. RNAs were denatured before annealing to the primer and extension reactions were carried out for 20 min at 52°C, followed by 5 min at 65°C. Capillary electrophoresis was carried out at the OSU Comprehensive Cancer Center Genomic Shared Resource facility. The data were processed using the QuShape software (56). SHAPE reactivity was derived from the difference between the frequencies of RT stops at each nucleotide in the presence and absence of NMIA. The data for each nucleotide were averaged after removing statistical outliers and normalized using the 2–8% rule (57).

Data availability: All data described are contained within the article.

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FIGURES

Figure 1. Predicted secondary structures of \textit{metI} S-box riboswitch. Helices P1-P4 form the aptamer domain. A) \textit{metI} sequence from \textit{D. indicum}. The sequence is shown in the sequestrator conformation. Green and red residues represent the alternate pairing required for the formation of the antisequestrator, shown above the sequestrator. The SD sequence is shown in blue. B) \textit{metI} sequence from \textit{B. subtilis}. The sequence is shown in the terminator conformation. Green and red residues represent the alternate pairing required for formation of the antiterminator, shown above the terminator.
Figure 2. SAM binding assays with metI riboswitches. (A) RNAs were incubated with increasing concentrations of \(^3\text{H}-\text{SAM}\) and filtered through size-exclusion filtration columns with a molecular weight cut-off of 10 kDa. The amount of RNA bound \(^3\text{H}-\text{SAM}\) retained on the filter was counted and the data were fit to a quadratic equation to determine the \(K_D\) values. ●, \(B.\ subtilis\) metI (red); ■, \(D.\ indicum\) metI (blue); ●, \(B.\ subtilis\) glyQS (grey). ND, no detectable binding (\(K_D > 200\ \mu\text{M}\)). (B). The wild-type (WT) and J1/2 mutant RNAs were incubated with \(^3\text{H}-\text{SAM}\). For the competition binding assays, RNA was incubated with \(^3\text{H}-\text{SAM}\) in the presence or absence of a competitor ligand (unlabeled SAM or SAH) at a 50-fold molar excess. Samples were passed through 10K size-exclusion filters and the RNA-bound \(^3\text{H}-\text{SAM}\) retained on the filters was counted. Error bars denote SD, \(n \geq 3\). Statistical differences in the fraction of \(^3\text{H}-\text{SAM}\)-bound WT RNA relative to the other reactions was determined by unpaired t-test. Significant = \(p<0.05\).

Figure 3. \(D.\ indicum\) metI RNA structural changes. (A) \(\alpha-^{32}\text{P}\)-labeled \(D.\ indicum\) metI RNA was annealed to an antisense oligonucleotide complementary to the SD region in the presence or absence of SAM, and incubated with RNase H. The products were resolved on a 6% denaturing polyacrylamide gel. FL, Full-length RNA; C, cleavage product. The solid line in the gel indicates that the reactions were loaded
in non-adjacent lanes in the same gel. (B) Primer extension inhibition analysis of the *D. indicum* *metI* RNA in the presence and absence of SAM. A γ-32P-labeled primer was annealed to the 3' end of the transcript and incubated with 30S ribosomal subunits and tRNA

in the presence or absence of SAM. Reverse transcriptase (RT) was added to initiate cDNA synthesis. The reaction products were resolved on a 6% denaturing polyacrylamide gel. The bands labeled as +16, +17 are RT stops that correspond to their positions with respect to the A of the AUG start codon in the transcript.

Figure 4. Stability of *metI* RNA-SAM complexes. (A). Aptamer domain of the *D. indicum* S-box riboswitch. A 2-aminopurine label was introduced at A14 of the P1 helix of the aptamer RNA (highlighted by pink circle). The break in the bipartite construct is shown between C60 and C61. Internal loop 3a (IL3a) and Internal loop 3b (IL3b) are highlighted in helix P3. Mutations introduced into IL3b are shown with arrows. Conserved adenines in IL3a that are a part of the SAM-binding core are shown in green. (B). Aptamer domain of the *B. subtilis* *metI* S-box riboswitch. G27 shown in lowercase depicts the residue introduced by T7 RNAP transcription. IL3a and IL3b are highlighted. Mutations introduced into IL3b are shown with arrows. Conserved adenines in IL3a that are a part of the SAM-binding core are shown in green. (C). Fluorescence-based kinetic experiments with the wild-type (blue) and mutant (red) *metI* translational RNA–SAM complexes. Equal volumes of preformed S-box RNA–SAM complex and 10-fold molar excess of the competitor *yitJ* leader RNA were rapidly mixed in a stopped-flow instrument and the fluorescence emission was monitored. Raw data are plotted as the fraction of SAM-bound RNA vs. time. The data were fit to single and double exponential equations to determine the dissociation rate constant (*k*off) and complex half-life. Data shown are average of at least 5 traces. (D). Size-exclusion
filtration-based kinetic experiments with wild-type (red) and mutant (blue) metI transcriptional RNA–SAM complexes. RNA was denatured, slow cooled, and incubated with $^3$H-SAM. Non-radioactive SAM was added at 100-fold molar excess as a competitor ligand. Samples were removed at intervals and were passed through BSA-treated Nanosep 10K filters. Complex half-life was determined by performing nonlinear regression analysis using GraphPad Prism 8. Error bars denote SD, $n \geq 3$.

**Figure 5. SHAPE analysis of RNA structure.** (A) Wild-type and (B) mutant translational metI RNAs. Residues in IL3b are highlighted (WT, blue; mutant, red). (C) Wild-type and (D) mutant transcriptional metI RNAs. Residues in IL3b are highlighted (WT, red; mutant, blue). RNAs were incubated with NMIA or DMSO, followed by an RT inhibition reaction. SHAPE reactivity is the difference between the frequency of RT stops at each nucleotide in NMIA vs. DMSO samples. Green bars show the conserved residues in IL3a and black arrows indicate significant changes in reactivity. The nucleotides are numbered below the x axis and the structural features are labeled on the top. Error bars denote SD, $n \geq 3$. 
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