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SAM Recognition and Conformational Switching Mechanism in the *Bacillus subtilis* *yitJ* S Box/SAM-I Riboswitch

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Abstract

S-box (SAM-I) riboswitches are a widespread class of riboswitches involved in the regulation of sulfur metabolism in Gram-positive bacteria. We report here the 3.0-Å crystal structure of the aptamer domain of the *Bacillus subtilis* *yitJ* S-box (SAM-I) riboswitch bound to *S*-adenosyl-L-methionine (SAM). The RNA folds into two sets of helical stacks spatially arranged by tertiary interactions including a K-turn and a pseudoknot at a four-way junction. The tertiary structure is further stabilized by metal coordination, extensive ribose zipper interactions, and SAM-mediated tertiary interactions. Despite structural differences in the peripheral regions, the SAM-binding core of the *B. subtilis* *yitJ* riboswitch is virtually superimposable with the previously determined *Thermoanaerobacter tengcongensis* *yitJ* riboswitch structure, suggesting that a highly conserved ligand-recognition mechanism is utilized by all S-box riboswitches. SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) chemical probing analysis further revealed that the alternative base-pairing element in the expression platform controls the conformational switching process. In the absence of SAM, the *apo yitJ* aptamer domain folds predominantly into a pre-binding conformation that resembles, but is not identical with, the SAM-bound state. We propose that SAM enters the ligand-binding site through the "J1/2–J3/4" gate and "locks" down the SAM-bound conformation through an induced-fit mechanism. Temperature-dependent SHAPE revealed that the tertiary interaction-stabilized SAM-binding core is extremely stable, likely due to the cooperative RNA folding behavior. Mutational studies revealed that certain modifications in the SAM-binding region result in loss of SAM binding and constitutive termination, which suggests that these mutations lock the RNA into a form that resembles the SAM-bound form in the absence of SAM.

Keywords

riboswitch; S-box; SAM-I; SHAPE; SAM

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Protein Data Bank: The coordinates for the *B. subtilis* *yitJ* riboswitch structures have been deposited in the Protein Data Bank with accession number 3NPB.

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Introduction

Riboswitches are short *cis*-acting RNAs that regulate gene expression in response to environmental cues (reviewed in Refs. 1–10). They usually reside in the 5' untranslated region of bacterial genes or operons and interact directly with effector molecules to regulate downstream gene expression at either the transcriptional or translational level. Bacteria use riboswitches to control vitamin metabolism, nucleotide and amino acid biosynthesis, sulfur metabolism, and metal transport. Rare examples of riboswitches that regulate alternative splicing or mRNA stability have also been found in eukaryotes.^{11,12}

Among more than a dozen distinct riboswitch classes that respond to various metabolites, the *S*-adenosylmethionine (SAM)-binding S-box (SAM-I) riboswitch class is arguably the most prevalent class,¹³ especially in Gram-positive bacterial genomes.¹⁴ S-box riboswitches were originally identified and extensively studied in *Bacillus subtilis*, where they control more than 26 genes involved in the import, synthesis, and recycling pathways for methionine or SAM.^{15–18} The S-box riboswitch is composed of a SAM-sensing “aptamer domain” followed by an “expression platform” to regulate downstream gene through transcription attenuation.^{15,16,18,19} Alternative base pairing between the aptamer domain and the expression platform is regulated by the intracellular concentration of SAM.^{16,20} Binding of SAM to the aptamer domain induces a tightly folded aptamer structure, which in turn allows the downstream formation of a transcription terminator to attenuate transcription in most S-box-regulated genes.^{2,16,17,19,20} The aptamer domain is destabilized in the absence of SAM, which allows the formation of a downstream antiterminator helix that allows RNA polymerase (RNAP) to read through the termination site and produce full-length transcripts. In addition, the SAM-IV class of riboswitches adopt secondary structures similar to that of the S box, but are proposed to regulate at the level of translation initiation; in this case, the terminator helix is replaced by an element that is predicted to sequester the ribosome binding site.²¹

S-box riboswitches in general are highly sensitive to and selective for the intracellular SAM pool. For example, the *B. subtilis* *yitJ* S-box riboswitch exhibits high affinity for SAM ($K_d \sim 20$ nM) and strongly discriminates against closely related natural analogues such as *S*-adenosyl-L-homocysteine (SAH; 100-fold lower) and *S*-adenosyl-L-cysteine (nearly 10,000-fold lower).^{16,18} Different S-box elements in *B. subtilis* vary widely in their sensitivity for SAM, despite the high conservation of the riboswitch sequence and predicted structure, with a 200-fold range of affinity for SAM; genes that are part of the core biosynthetic pathway generally show higher sensitivity than genes encoding methionine transporters, resulting in a hierarchical response to changing SAM pools.²⁰

Phylogenetic and mutagenesis analyses revealed that the S-box aptamer domain consists of four helical segments organized around a four-way junction, and P2 and P4 were shown to participate in formation of a pseudoknot.^{15,17,22} The crystal structure of a 94-nt *yitJ* SAM-I (S box) riboswitch from the thermophilic bacterium *Thermoanaerobacter tengcongensis* has been reported.^{23,24} It showed that the phylogenetically conserved helices organize into two sets of coaxial helices packed against each other at a tilted angle and enclosing SAM at the helical packing interface.^{23,24} Mutagenesis studies on the *T. tengcongensis* *yitJ* riboswitch further confirmed that the riboswitch distinguishes SAM from SAH by specifying the positively charged sulfonium ion in SAM.²⁴ Recent structural and biochemical studies on the *apo* *T. tengcongensis* *yitJ* riboswitch aptamer domain revealed a number of closely related, pre-organized conformational states in the presence of magnesium ion,²⁵ suggesting the presence of a “sensing phase” during transcription and riboswitch folding. In this study, we combine crystal structure determination, selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) chemical probing, and mutagenesis to study the *B. subtilis* *yitJ* S-

box aptamer RNA, which is the S-box riboswitch that has been most carefully characterized in biochemical and genetic studies.^{15,16,17,22} Our structure revealed that the ligand-recognition mechanism is highly conserved among the S-box riboswitch family. We further conclude that the alternative base-pairing element in the expression platform controls the conformational switching process, and SAM is recruited to the ligand-binding site through the “J1/2–J3/4” gate and “locks” the S-box SAM-binding core through an induced-fit mechanism.

Results

Comparison of the *B. subtilis* and *T. tengcongensis* *yitJ* riboswitch structures

The overall architecture of the 119-nt *B. subtilis* *yitJ* S-box RNA is similar to that of the 94-nt *T. tengcongensis* riboswitch (PDB code: 2GIS, 3GX5),^{23,24} despite a large deviation in the equivalent phosphorous atoms (r.m.s.d.=2.5 Å; Fig. 1a). Similar to the *T. tengcongensis* *yitJ* RNA, the four helices in the *B. subtilis* *yitJ* S-box aptamer assemble into two sets of coaxial helices, P1/P3 and P2/P4 (Fig. 1b). These superhelices then pack against each other at an ~70° tilted angle through extensive minor-groove ribose zipper interactions (described below), a K-turn motif in the middle of P2 that bends its distal portion ~120° to wrap around P1/P3, and a pseudoknot in the distal loop of P2 and J3/4 that weaves the P1/P3 and P2/P4 into a rigid conformation. The distal portion of the P3 helix, which is significantly longer in the *B. subtilis* *yitJ* structure, packs against the P4 helix at an average distance of 6 Å (Fig. 1b).

The mesophilic *B. subtilis* *yitJ* structure displays a wide distribution of temperature *B*-factors for each nucleotide, which is a good indicator of local thermal motion/conformational flexibility in the RNA (Supplemental Fig. 1). This is in sharp contrast to the relatively even distribution of *B*-factors in the thermophilic *T. tengcongensis* *yitJ* structure^{23,24} and likely reflects the adaptation of each riboswitch to its native environment. In the *B. subtilis* *yitJ* structure, nucleotides at the SAM-binding core and the SAM molecule itself exhibit a much lower average temperature factor ($B_{\text{AVG}}=35$) than that of the overall structure ($B_{\text{AVG}}=87$; Supplemental Fig. 1). Certain peripheral regions of the *B. subtilis* *yitJ* RNA, such as those in the loop of P4 and J2/3, display significantly higher temperature factors (>150; Supplemental Fig. 1). While the absolute temperature factor is impacted by both thermal motion and the order of the crystal lattice, the relative distribution of *B*-factors is a good indicator of the thermal motions within a macromolecular structure. Therefore, higher overall *B*-factors in the peripheral regions (including the P4 terminal loop, distal P1, and the K-turn region in P2) suggest that these regions are much more flexible than the SAM-binding core. As shown later, this conclusion is in good agreement with that obtained from a temperature-dependent SHAPE analysis.

The *B. subtilis* *yitJ* SAM-binding pocket is almost superimposable with that of the *T. tengcongensis* *yitJ* riboswitch

While significant structural differences can be found in the peripheral regions of the two S-box riboswitches, the *B. subtilis* *yitJ* RNA geometry at its SAM-binding core displays striking similarity to the *T. tengcongensis* *yitJ* RNA (r.m.s.d. of 0.37 Å for alignment of the equivalent phosphorous atoms), suggesting that riboswitches in this family use a conserved mechanism to recognize SAM. Inside the *B. subtilis* *yitJ* SAM-binding pocket, SAM adopts the same compact, U-shaped conformation seldom seen in protein-bound structures to allow the parallel stacking of its methionine moiety on top of the adenosine ring (Fig. 2a). The Watson–Crick and Hoogsteen edges of the adenosine base of SAM are collectively recognized by four hydrogen bonds in a universally conserved, sheared A46-U78 pair (Fig. 2b). The methionine moiety of SAM makes four hydrogen bond contacts with the G79-

C45-G11 base triple, which is part of the structure responsible for tying together J1/2 and P3 (Fig. 2c).

The positively charged sulfonium group in SAM is specified by a pair of electrostatic interactions with the O4 carbonyl oxygen of U7 and U112 (3.5 and 4.1 Å away, respectively; Fig. 2d, distances shown in magenta). This arrangement is similar to that in the wild-type *T. tengcongensis* *yitJ* RNA structure²³ and almost identical with that in the A94G/U34C *T. tengcongensis* *yitJ* mutant. The importance of this electrostatic interaction has been demonstrated previously by U7C and U112C mutations, which eliminate the two interacting carbonyl groups and severely impaired SAM binding in *T. tengcongensis* *yitJ* RNA.²⁴ In addition to the RNA–SAM interactions, the sulfonium ion makes a very strong ~3.0-Å intramolecular electrostatic interaction with the O4' of the ribose in SAM (Fig. 2d, distance shown in cyan).^{26,27} The methyl group on SAM does not directly contact the riboswitch and orients toward the solvent. Comparison of the S-box structures to those of the SAM-II and S_{MK} box (SAM-III) SAM-binding riboswitches (Refs. 23,24,26,27 and this study) demonstrates that all three classes of SAM riboswitches use a similar mechanism to selectively recognize the positively charged sulfonium ion in SAM and discriminate against the near-cognate ligand SAH, which bears a neutral sulfide in place of sulfonium ion, via favorable inter- and intra-molecular electrostatic interactions with the sulfonium ion, without direct contact with the S-methyl group. There is no conserved specific contact for the C^β and C^γ atoms in the methionine moiety of SAM, and their corresponding electron densities are significantly weaker due to thermal motions.

A set of conserved tertiary interactions zip up the SAM-binding site

In addition to the K-turn and pseudoknot interactions previously reported in the *T. tengcongensis* *yitJ* structure (and previously predicted by phylogenetic, genetic and biochemical analyses^{15,17,22}), we report additional sets of tertiary interactions that stabilize the *B. subtilis* *yitJ* tertiary structure, apparently independent of the SAM molecule (Fig. 3a and b). Surrounding the SAM-binding pocket are several clusters of conserved ribose zippers (Fig. 3a, red bases). One set of these interactions is responsible for adenine Hoogsteen face recognition and defines the back and right side of the SAM-binding pocket, involving the ribose between nucleotides (nt) 112–114 of P1 and nt 77–80 of P3 (Fig. 3b, blue strands). A similar set of ribose zippers are above the methionine portion of the SAM-binding pocket (Fig. 3b, red strands), where interactions between G44, C45, G11, and A12 weave J1/2 together with J3/4 and position G11 for recognition of the carboxyl group in SAM. A magnesium ion, together with J1/2 and J3/4, seals the methionine side of the binding pocket (left side as shown in Fig. 3b), and our SHAPE probing data suggest that this side of the binding pocket is likely to be important for SAM recruitment in the *B. subtilis* *yitJ* RNA (see below). Last but not least, the conserved C48 residue makes two hydrogen bonds through its O2 and O2' with A113, closing off the adenosine side of the binding pocket. This extensive network of ribose-mediated hydrogen bonds seals the back, top, right, and left portions of the binding pocket in an apparently SAM-independent fashion, suggesting that the aptamer domain of the *B. subtilis* *yitJ* S-box RNA may pre-assemble into a conformation similar to that observed in the crystal structure in a SAM-independent manner.

In addition to stabilizing the SAM-binding pocket, ribose-mediated hydrogen bonds also help to stabilize the P2/P4 pseudoknot (Fig. 3b, cyan bases). The A84-C86 turn directly positions A83 and C86 for pseudoknot base pairing, while the Watson–Crick face of A82 interacts with O2' and O2 of C30 in the distal P2 loop. O2' of A109 interacts with N1 of A24, further stabilizing the pseudoknot. These SAM-independent interactions are also present in the *T. tengcongensis* *yitJ* structure, but are not discussed in this article.^{23,24}

A magnesium ion mediates key ligand-induced conformational changes

Two inner-sphere coordinated magnesium ions (Ma and Mb) were assigned with confidence in our 3.0-Å resolution *B. subtilis* *yitJ* S-box structure. One of the magnesium ions, Ma, is located near the methionine tail side of the SAM-binding pocket and makes two inner-spherical coordination with phosphoryl oxygen atoms from A10 and U65, as well as four outer-spherical interactions with N7 of A10, O6 and N7 of G11, and the phosphoryl oxygen of A83 (Fig. 4). Ma serves the important function of tying together the J3/4 U-turn and J1/2 to stabilize the coaxial stacking of P1 and P4 and facilitates formation of the pseudoknot by precisely orienting three of the four pseudoknot base pairs. The Ma site is conserved in the two *yitJ* riboswitch structures^{23,24}; a divalent ion binds to the equivalent location in the *T. tengcongensis* *yitJ* riboswitch and makes the identical interactions with surrounding residues including the conserved A10 and G11 residues (Fig. 4). Interestingly, A10 appears to be conserved solely for the purpose of participating in the Ma binding pocket. This magnesium-mediated junction in the *B. subtilis* *yitJ* riboswitch also contains an A9/A84 dinucleotide stack, which is not evident in any of the *T. tengcongensis* *yitJ* riboswitch structures.^{23,24} Our SHAPE probing data (see below) suggest that this metal-ion site could potentially serve as a door to the SAM entry site and secure the SAM pocket with J1/2 and J3/4 synergistically as the door is closed. This result is in accordance with recent studies on the *apo T. tengcongensis* *yitJ* riboswitch,²⁵ which requires sufficient divalent magnesium ion to fold into ligand-sensing tertiary structures. Such metal-induced tertiary interactions are prevalent in riboswitches, as also recently illustrated in the *apo* TPP riboswitch.²⁸

The S-box aptamer adopts a pre-binding conformation that is revealed upon uncoupling from the expression platform

A key open question is the nature of the conformational switching mechanism that is coupled with the ligand-recognition event. To address this, SHAPE analysis, which detects local RNA flexibility through the extent of ribose 2'-OH alkylation, was used to compare the conformation of the *B. subtilis* *yitJ* RNA, with or without the 3' expression platform (i.e., the terminator sequence), in the presence or absence of SAM.

In the first set of experiments, the aptamer domain of the *B. subtilis* *yitJ* S box was subjected to SHAPE probing in the presence or absence of SAM. The SHAPE reactivity of the SAM-bound aptamer domain correlates very well with our crystal structure, in that the residues involved in secondary and tertiary structure formations all displayed low SHAPE reactivity, whereas distal loops and single-stranded residues in the P2 K-turn (G19, A20, A33), P3 bulge (G53-A55), and P4 terminal loop (C96) all displayed higher-than-average SHAPE reactivity. These results indicate that our crystal structure is a good depiction of the SAM-bound S-box aptamer domain conformation in solution. Interestingly, SHAPE analysis also revealed that the S-box aptamer domain conformation in the absence of SAM was largely similar to the SAM-bound conformation. This well-folded “pre-binding” conformation, however, contained noticeable differences in discrete regions, most prominent at J1/2, J3/4, and nt A46-A47. These residues are involved in either critical SAM-dependent tertiary contacts (J1/2, J3/4) or direct SAM recognition (nt A46-A47; Fig. 5, right panel). The J1/2 and J3/4 data are particularly interesting, as they suggest the presence of a J1/2-J3/4 gate that is open in the absence of SAM but closes in the presence of SAM. The overall similarity between the pre-binding and liganded aptamer conformations strongly suggests that in the absence of the expression platform, SAM-independent tertiary interactions are strong enough to pre-organize the S-box aptamer domain into a well-folded state largely ready for ligand binding. The discrete conformational differences between the pre-binding and SAM-bound states revealed further conformational changes induced by SAM binding, which suggests that ligand recognition is likely to utilize an induced-fit mechanism. Since the folding of RNA is generally several orders of magnitude faster than transcription

(milliseconds *versus* seconds),^{29–31} the likely scenario is that the *B. subtilis* *yitJ* S-box aptamer domain adopts a pre-binding conformation before the terminator sequence is transcribed, thereby providing a window of opportunity for SAM binding to attenuate transcription (see Discussion).

A parallel set of SHAPE probing experiments was carried out on full-length *B. subtilis* *yitJ* riboswitch RNA, including the expression platform. The results suggested that the presence of SAM induces the formation of a well-folded aptamer domain and the terminator stem-loop, consistent with its role in transcription attenuation. In the absence of SAM, however, a drastically less protected reactivity pattern was found in the aptamer domain of the full-length *yitJ* RNA (Fig. 5, left panel, lane 2). This was especially apparent at the P1 stem, J1/2, P2 K-turn, J2/3, J3/4, part of P3, and P4 pseudoknot. Downstream of the expression platform region, SHAPE revealed that the transcription terminator stem-loop was replaced by a rather flexible structure pattern in the absence of SAM. We could not clearly define the transcription antiterminator stem-loop, but it is likely that the SHAPE reactivity reflects partial formation of this element¹⁵ (data not shown). Therefore, we propose that SAM-induced conformational switching is dependent on the presence of the expression platform, which alters the free-energy folding landscape such that in the absence of SAM, an alternatively base-paired conformation involving the participation of the expression platform is significantly more stable than the pre-binding aptamer conformation. As a result, the presence of the expression platform in the RNA strongly influences the folding pattern of the aptamer domain in the absence of SAM.

Mutational analysis of the *B. subtilis* *yitJ* riboswitch

Structural predictions were tested by mutation of key residues and testing for effects on SAM binding and transcription termination *in vitro*. Mutation of residue G11, which participates in the G79-C45-G11 base triple that ties together J1/2 and P3 and contacts the methionine moiety to A, C, or U, resulted in loss of detectable SAM binding (Table 1), consistent with its position in the crystal structure. Surprisingly, each of these mutations resulted in increased transcription termination in the absence of SAM, with the most dramatic effect exhibited by the G11C mutant, which exhibited >80% termination in the presence or absence of high SAM (140 μ M; in contrast, the wild-type construct exhibits a maximal response at 0.2 μ M SAM). The high termination efficiency in the absence of SAM suggests that alteration of G11 facilitates formation of an RNA structure resembling the SAM-bound form even in the absence of SAM. The G11A substitution had only a modest effect on termination in the absence of SAM, despite complete loss of SAM binding, indicating that the structural change that results in the stabilization of RNA into a form similar to the ligand-bound form is separable from the effects on SAM binding. Mutations of the other residues that participate in the base triple (G79 and C45) had a similar effect, suggesting that any disruption of the base triple causes a surprising stabilization of the aptamer domain structure.

The A46-U78 pair, which forms a base triple with the adenine ring of SAM, was also highly sensitive to mutation and exhibited a pattern similar to that of the G79-C45-G11 region (Table 1). Mutation of the A47-C48-G77 residues that further stabilize the adenine ring of SAM also resulted in high termination in the absence of SAM; however, several substitutions at these positions (notably C48A and C48G) retained a partial response to high SAM, indicating that SAM binding was not affected as strongly. In contrast, mutation of the U85-A109 pair that supports the pseudoknot did not significantly affect termination in the absence of SAM. Combination of the U85C and A138G mutations did not suppress the effect of either single mutation, which shows that these residues exhibit sequence specificity in addition to their ability to base pair, consistent with the role of A139 in the formation of a base triple with A24 adjacent to the pseudoknot.

We also tested the effect of mutations at the U7-A111 pair in the P1 helix, which recognizes the sulfonium ion. An A111G mutation, which places a U-G pair at this position, did not significantly affect termination in the absence of SAM and did not obliterate the response to SAM, although the affinity for SAM was greatly reduced (>200 -fold increase in K_d ; Table 1). Replacement of the U-A pair with a G-U (U7G/A111U) resulted in a response similar to that of the A111G mutant, although the affinity for SAM was approximately 10-fold higher. In contrast, the U7G/A111C mutant, in which the U-A pair is replaced with a G-C, exhibited high termination in the absence of SAM, but SAM binding was reduced only 20-fold relative to wild-type. In this case, the mutation may simply stabilize the P1 helix, thereby inhibiting antiterminator formation and promoting termination. Stabilization of an RNA conformation similar to the SAM-bound form is therefore separable from the effects on SAM binding, as noted above for the G11A mutation.

Thermal melting of the S-box RNA bound to SAM reveals differential stability of structural elements

The SAM-bound S-box RNA is a compact, well-ordered structure that encompasses many tertiary interactions such as a pseudoknot, ribose zippers, Mg-mediated strand couplings, as well as SAM-dependent tertiary interactions. Therefore, it is likely that the folding/refolding process of the SAM-bound S-box RNA is highly hierarchical. We employed a temperature-dependent SHAPE analysis to obtain thermal stability information for the *B. subtilis yitJ* riboswitch to study differential stability among the different domains of the RNA.

The *B. subtilis yitJ* SAM-bound S-box aptamer domain displays remarkable thermal stability, as almost all of the tertiary interactions remain intact even at 50 °C. Toward the higher end of the temperature gradient, about 80% of the structure became more flexible (Fig. 6a). However, in contrast to the extremely stable thermophilic *T. tengcongensis yitJ* RNA,²⁸ the *B. subtilis yitJ* riboswitch showed differential melting points among its structural elements. The temperature melting behavior of individual bases was quantified and clustered according to the secondary structures in which they reside. Surprisingly, many tertiary structures appear to be more stable than the secondary structures. For example, helices P1 and P4 melted at intermediate temperatures, with a transition T_m of ~56 °C. In contrast, J3/4 unfolded with a high transition T_m of ~58 °C; the pseudoknot structure unfolded with an even higher transition T_m of 65 °C; and J1/2 and J2/3 did not show signs of unfolding at the higher temperature range of 70 °C (Fig. 6b). The hierarchical melting pathway of the *B. subtilis yitJ* S-box riboswitch is different from that of tRNA, in which the tertiary interactions disintegrate before the secondary structure.³² Here, the strong tertiary interactions responsible for stabilizing the *B. subtilis yitJ* aptamer core, both ligand induced (e.g., J1/2 and J2/3) and ligand independent (e.g., the pseudoknot), remain intact before some of the peripheral secondary structure elements (e.g., the P1 helix) disintegrate. Despite the relatively earlier melting of peripheral helices, the SAM-binding core is likely to be held together by several individual bases in P1 and P4 that appeared to unfold at much higher T_m (marked with asterisk in Fig. 6, left panel). This observation is consistent with the structural analysis showing that the nucleotide temperature B -factors in the SAM-binding core are much lower than that in the peripheral region (Figs. 2b and 6b, upper panels). Assuming that folding of the *B. subtilis yitJ* aptamer domain is thermodynamically reversible, our results also suggest that folding of the *B. subtilis yitJ* riboswitch is cooperative and that many of the tertiary interactions form first due to higher stability and possibly guide the formation of some of the secondary structures. This folding scheme seems to be consistent with a 2-AP study,³³ which showed that the pseudoknot and K-turn structure guide the folding of the *B. subtilis yitJ* riboswitch.

Discussion

Through comparison of *B. subtilis* and *T. tengcongensis* *yitJ* riboswitch structures, we conclude that despite the differences in their surface and peripheral regions, they utilize a virtually identical mechanism to recognize the SAM molecule because the conformations of SAM and surrounding RNA residues involved in SAM recognition are virtually superimposable. We also revealed that conserved SAM-dependent and SAM-independent tertiary interactions are likely to play important roles in stabilizing the structural integrity as well as the conformational dynamics. The full-length riboswitch, containing the aptamer domain and the expression platform, clearly switches its conformation in a SAM-dependent fashion. In contrast, in the absence of the expression platform, the aptamer domain of the *B. subtilis* *yitJ* riboswitch folds into a SAM-bound-like conformation even without SAM. A similar result was observed from the *apo* *T. tengcongensis* *yitJ* aptamer domain, which samples several defined tertiary structures similar to that of the SAM-bound state.²⁵ This suggests that the SAM-independent interactions noted above are strong enough to direct the folding of the aptamer domain into a pre-binding conformation. We propose that this pre-binding conformation has a pre-organized binding pocket, with an open gate at the J1/2–J3/4 region near the methionine side of the SAM-binding pocket. SAM entry is coupled with closure of the J1/2–J3/4 gate, which induces the SAM-bound conformation by chelating a divalent ion (Mg) and positioning G11 in J1/2 for SAM recognition. This hypothesis is supported not only by the structural identification of SAM-independent tertiary interactions but also by the SHAPE analysis revealing localized conformational movement in J1/2–J3/4 in the *B. subtilis* *yitJ* aptamer domain. In addition, formation of the pre-binding state is consistent with molecular dynamics simulations³⁴ and structural and biochemical studies²⁸ performed on the aptamer domain of the *T. tengcongensis* and *B. subtilis* *yitJ* riboswitch, which did not detect a globally unstructured *yitJ* aptamer domain conformation in the absence of SAM.

We also characterized a set of mutations within the SAM-binding domain. Most of these mutations disrupted SAM binding, as expected for mutations of residues that make important contacts with SAM or that are important for stabilization of crucial structural domains that form the SAM-binding pocket. The more surprising mutations were those that resulted in high transcription termination in the absence of SAM, suggesting that they may stabilize a structural arrangement in the aptamer domain similar to the SAM-bound form. Several of these mutants retained the ability to respond to SAM, indicating that formation of this conformation in the absence of SAM is separable from the ability to bind SAM. A better understanding of how these mutations preferentially stabilize the pre-binding state awaits further structural analysis.

In summary, our chemical probing data are consistent with a transcription-dependent conformational switching model for the *B. subtilis* *yitJ* S-box riboswitch (Supplemental Fig. 2). RNAP first transcribes the aptamer domain of the S-box RNA, which immediately folds into the pre-binding conformation before transcription of the downstream terminator sequence. It was estimated that it takes less than 100 ms for the riboswitch to fold,^{29,30} compared to the transcription rate of ~40 nt/s by bacterial RNAP.³¹ Therefore, the aptamer domain of *yitJ* has ample time to fold into the pre-binding conformation before RNAP transcribes the expression platform. This pre-binding aptamer conformation has two alternative fates: (1) If the intracellular SAM level is high, the aptamer structure is greatly stabilized by SAM-mediated tertiary interactions and cannot be competed away by the subsequent emergence from RNAP of the antiterminator sequence, leading to terminator hairpin formation and transcription termination. (2) If the intracellular SAM level is low, the aptamer structure collapses owing to competing base-pair formation from the expression platform; the antiterminator hairpin forms, which sequesters sequences that would otherwise

participate in the formation of the terminator helix; and transcription continues to produce the full-length transcript (Supplemental Fig. 2). Either decision by the RNAP is irreversible; therefore, S-box transcription riboswitches are not under selection pressure to be able to switch from the SAM-bound to the unfolded conformation. The *B. subtilis yitJ* S-box RNA–SAM complex is very stable *in vitro*, with a half-life of >7 min,²⁰ consistent with the model that folding of the RNA into the SAM-free *versus* SAM-bound form as it emerges from RNAP represents a brief opportunity for the ligand to bind and dictate whether RNAP will terminate or continue past the termination site. This “window-of-opportunity” model would not work if the rate of transcription is faster than that of aptamer domain folding.

The observation that certain sequence changes within the aptamer domain not only block SAM binding but also promote formation of a structure that resembles the SAM bound form is similar to previous observations with the Thi-box riboswitch.³⁵ The identification of mutations of this type indicates that the evolution of a riboswitch capable of an effective regulatory response is subject to multiple constraints. The aptamer domain must have binding affinity appropriate to the physiological pools of the ligand that is detected and selectivity against biologically relevant analogues. Regulatory function also requires that the aptamer domain folds into a conformation in the absence of ligand that is capable of ligand recognition but fails to completely mimic the ligand-bound form, allowing a ligand-dependent structural rearrangement that can be coupled to gene regulation. Subtle sequence changes that cause the RNA to shift into a conformation resembling the ligand-bound form will inappropriately trigger the ligand-dependent gene regulation response.

Materials and Methods

RNA preparation for crystallization

The aptamer domain of the *B. subtilis yitJ* S-box riboswitch (nt 29–146) was inserted into a pUC19 plasmid under the control of a T7 RNAP promoter; the hepatitis delta virus ribozyme was positioned at the 3'-end to ensure a homogeneous 3'-endpoint after ribozyme cleavage. RNA was prepared by a standard T7 RNAP run-off transcription reaction using linearized plasmid DNA as template and purified by denaturing gel electrophoresis as described.³⁶ SAM was purchased from Sigma and stored as a 100 mM stock solution at pH 6.0 at –80 °C until immediately before use.

Before crystallization, the RNA was refolded by heating up to 65 °C for 10 min in a buffer containing 25 mM Tris–HCl (pH 7.5), 40 mM NaCl, and 5 mM MgCl₂, at which point SAM was added to a final concentration of 1 mM. The solution was allowed to cool gradually to 20 °C over a course of 1 h and stored frozen at –80 °C. The wild-type S-box aptamer domain constructs produced crystals that diffracted only to ~6 Å resolution. The distal P3 loop was found to have undergone spontaneous cleavage during crystallization (data not shown). This observation led to the design of three constructs in which the flexible stem-loop was replaced with a stable GAAA tetraloop and the base pairs below the tetraloop were systematically shortened; these constructs retained wild-type SAM binding activity. One of the resulting constructs, designated TL5, produced a rare crystal form [in 40 mM sodium cacodylate (pH 7.0), 80 mM strontium chloride, 15% methyl-2,4-pentanediol, and 2 mM spermine] after prolonged incubation, which diffracted X-rays to a resolution of ~3 Å. The crystals were soaked in increasing concentrations of methyl-2,4-pentanediol (up to 25%) in the presence of 1 mM freshly prepared SAM and then flash-frozen in liquid nitrogen.

Molecular replacement and structure refinement

A 3-Å data set was collected from a TL5 crystal at beamline F1 at Cornell High Energy Synchrotron Source (CHESS). We solved the structure with the molecular replacement

program PHASER,³⁷ using the core *T. tengcongensis* *yitJ* structure²³ (PDB code: 2GIS), excluding SAM and the distal portion of the P3 helix, as the search model. The P3 helix was later located by PHASER³⁷ using a 17-bp ideal A-form helix capped by a GAAA tetraloop generated by COOT.³⁸ Repeated rounds of density modification with model-phase inputs, rigid-body refinement, and harmonically restrained simulated annealing refinement procedures in CNS^{39,40} reduced the initial-phase errors and allowed tracing of most of the sugar-phosphate backbone (Table 2). Phylogenetically conserved base pairing was reinforced by specifying Watson-Crick base pairs throughout CNS refinement.^{39,40} To further reduce the model bias and phase errors, the original search model was refined for several rounds as a poly-cytosine model, which produced an electron density map of sufficient quality to distinguish purines from pyrimidines. This map allowed accurate modeling of bases in COOT³⁸ and restrained refinement in Refmac5⁴¹ until the model was ~93% complete and R_{free} was below 35%. The complete model was generated after iterative cycles of group *B*-factor refinement, energy minimization, and simulated annealing in CNS.^{39,40} The SAM molecule, two divalent metal ions, and eight water molecules were identified in the $F_o - F_c$ difference density map and refined using real-space fitting in COOT.³⁸ The entire structure was verified using simulated annealing composite-omit maps by excluding 5% of the model in each calculation.^{39,40} The final model includes all but four flipped out nucleotides in the P3 stem and the P4 loop with a $R_{\text{work}}/R_{\text{free}}$ of 0.236/0.286.

RNA preparation for SHAPE analysis

The *B. subtilis* *yitJ* S-box RNA with or without the 3' expression platform (containing the complete antiterminator and terminator sequences) was inserted into a SHAPE cassette containing a 5' T7 RNAP promoter and a 3'-reverse transcription primer site by overlapping PCR and then cloned into a pJet cloning vector (from Fermentas). After sequence verification, the SHAPE cassette DNA templates were PCR amplified using terminal primers and used directly to produce RNAs for SHAPE analysis by *in vitro* transcription as described.²⁷ The RNA product was purified and recovered by denaturing polyacrylamide gel electrophoresis as described.³⁶ All RNAs were subsequently diluted into RNA folding buffer [111 mM Hepes (pH 8), 6.7 mM MgCl_2 , 111 mM NaCl] to ~0.5 μM . Immediately prior to chemical probing, the diluted RNA was refolded as previously described²⁷ in the presence or absence of SAM during the annealing process.

SHAPE chemical probing analysis

SHAPE chemical probing was performed as described previously.^{32,42,43} Using temperature-dependent SHAPE analysis as an example, 15 μL of the S-box RNA (0.3–1.0 μM , refolded $\pm$ SAM) was pre-equilibrated using a gradient PCR machine to the desired temperature (20, 30.0, 33.8, 43.9, 48.4, 50.0, 52.0, 53.3, 58.7, 58.9, 63.0, 66.4, or 68.6 °C) for 1 min and subsequently mixed with 1.5 μL of 100 mM electrophile 1M7 to initiate 2'-ribose hydroxyl alkylation. The reactions were allowed to proceed to completion at each temperature point by incubating for five half-lives of 1M7^{32,44}; the reaction was then quenched with 900 μL of precipitation buffer containing 80% EtOH, 45 μM NaCl, 0.45 μM EDTA (ethylenediaminetetraacetic acid), and 2 μL glycoblue (15 mg/mL; Ambion, Inc). The RNA was precipitated by incubation at –80 °C for 30 min, followed by centrifugation at 16,000g at 4 °C for 45 min. The pellet was Air-dried and resuspended in 12 μL of 0.5 $\times$ TE buffer.

The primer extension protocol was similar to that described previously.⁴² A fluorescently labeled DNA primer (HEX-GAACCGGACCGAAGCCCG; 1 μL , 50 μM) was annealed to the recovered RNA (12 μL) by incubation at 65 °C for 2 min, then 35 °C for 5 min, followed by snap cooling to 4 °C for another 2 min. The resulting 13 μL of RNA-primer mixture was mixed with 6 μL of reverse transcription buffer [167 mM Tris (pH 8.3), 250 mM KCl, 10

mM MgCl₂, 1.67 mM of each dNTP], preheated to 52 °C for 45 s, and then incubated with 1 µL of Superscript III (Invitrogen, 200 units) at 52 °C for 20 min. The reactions were quenched, and the RNA template was destroyed by addition of 0.8 µL of 5 M NaOH, followed by heating to 90 °C for 4 min. Acid stop mix [4:25 (v/v) mixture of 1 M unbuffered Tris–HCl and stop solution (85% formamide, 0.5× TBE, 50 mM EDTA, pH 8.0); 29 µL] was added to each reaction; the reaction was incubated for an additional 4 min at 90 °C and then cooled to –20 °C. No internal tracking dye was used in the reaction to avoid interference with fragment analysis.

SHAPE data analysis

Each set of primer extension reactions was evaluated by polyacrylamide sequencing gel electrophoresis and capillary electrophoresis/fragment analysis. To visualize band intensities, 5–10 µL of each reverse transcription reaction was separately loaded onto lanes of 10% denaturing sequencing gels (10% 29:1 acrylamide/bis acrylamide, 1× TBE, 8 M urea) and run at a constant voltage of 55 W. In order to obtain optimal separation for both high and low molecular weight fragments, we performed two scans from a Typhoon phosphor-imager (Molecular Dynamics) for every gel image. The first image for low molecular weight bands was scanned after 2.5 h of electrophoresis, and a second image for higher molecular weight fragments was obtained after 5 h of electrophoresis.

Our quantification analysis used results from fragment analysis (Applied BioSystems 3730xl DNA Analyzer) to determine intensity differences at the regions of interest. Traces from fragment analysis were quantified by the program ShapeFinder.⁴⁵ Nucleotide reactivities were scaled to uniformly reactive nucleotides at loop positions (G61–A63 for nt U1–C66; A96–C100 for A68–C118). Strong signals possibly caused by pausing were manually eliminated from the data. For temperature melting data, intensities from several key regions (J1/2, J2/3, pseudoknot, J3/4, and P1+P4) were rescaled to a unit (0 to 1) and fitted to the following equation, assuming a unimolecular transition,⁴⁶ using the program Origin (Origin Lab).

$$I = A \frac{1}{1 + \left[\exp \left[\frac{H_{\text{MH}}}{R} \left(\frac{1}{T_{\text{m}}} - \frac{1}{T} \right) \right] \right]} + b$$

where I is SHAPE activity at a given temperature (T); R is the gas constant; and A and b are the maximum and initial intensity, respectively. Since it is difficult to get a consistent behavior from a single base, the resulting melting curve is an averaged melting profile for a domain of interest.

Mutational analysis

Mutations in the *B. subtilis* *yitJ* S-box riboswitch were generated by oligonucleotide-directed mutagenesis as previously described.¹⁶ Templates for *in vitro* transcription assay were PCR fragments in which a *B. subtilis* *glyQS* promoter was positioned upstream of the *B. subtilis* *yitJ* leader sequence; template DNA extended past the termination site so that terminated and readthrough transcription products could be resolved easily on a 6% denaturing polyacrylamide gel. Templates were transcribed using *B. subtilis* RNAP in the presence or absence of SAM (140 µM) as previously described.^{16,20} Percent termination was calculated as the amount of terminated product relative to the total of terminated and readthrough products after Phosphor-Imager analysis. All assays were carried out in triplicate, and values shown are the average of three independent trials; variance was ±5%.

RNA was prepared for SAM-binding assays by positioning the *yitJ* leader sequence downstream of a T7 RNAP promoter and carrying out T7 RNAP transcription and RNA purification as described above. RNAs were heated and slow-cooled in 1× transcription buffer containing 10 mM MgCl₂, in the presence or absence of ³H-SAM; free SAM was separated from RNA-bound SAM by filtration and quantified for *K_d* determination as previously described.²⁰ All binding assays were carried out in triplicate, and error was ±5%.

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Abbreviations used

SAM	<i>S</i> -adenosyl- <i>L</i> -methionine
RNAP	RNA polymerase
SAH	S-adenosyl- <i>L</i> -homocysteine
CHESS	Cornell High Energy Synchrotron Source
SHAPE	selective 2'-hydroxyl acylation analyzed by primer extension

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Fig. 1.

Crystal structure of S-box (SAM-I) RNAs bound to SAM. (a) Phosphorous alignment of the structure between the *B. subtilis* (gray) and *T. tengcongensis yitJ* riboswitches (blue).²³ (r.m.s.d.=2.5 Å) (b) Secondary structure diagrams of the *B. subtilis yitJ* riboswitch. Helices P1 through P4 are colored in gray, green, cyan, and yellow, respectively. Red dashes denote pseudoknot base pairs. SAM-contacting bases are labeled in magenta. The sheared A46-U78 base pair that recognizes the adenosine base of SAM is labeled with a red dot. (c) Ribbon representations of the *B. subtilis yitJ* RNA structure. The two views represent a front view (left) and a 90° rotation (right). The color scheme is the same as that in (b). SAM is shown as a ball-and-stick model overlaid with surface representations in gray. Two assigned magnesium metal ions are shown in pink.

Fig. 2.

SAM-binding pocket of the *B. subtilis yitJ* riboswitch. (a) The U-shaped SAM molecule sits at the four-way junction between J2/3 and J1/2. (b) A sheared A46·U78 pair recognizes the Watson–Crick and Hoogsteen faces of the adenosine moiety of SAM. (c) The G79·C45·G11 base triple recognizes the methionine tail of SAM. (d) Stereo view of the electrostatic interactions between U7/U112 with the sulfonium ion. Also note the ~3.0-Å intramolecular interaction between the sulfonium ion and O4' of the ribose moiety of SAM. All labeling and color schemes are the same as those in Fig. 1b and c. The 3.0-Å simulated annealing omit map is contoured at 1.0 σ . Distances are given in angstrom in magenta. Carbon, oxygen, nitrogen, sulfur, and phosphorus atoms are colored gray, red, blue, gold, and orange, respectively.

Fig. 3. Stereo view of important SAM-independent tertiary interactions within the *B. subtilis yitJ* RNA. The orientation of the S-box RNA structure is the same as that in Fig. 2. In this view, the SAM molecule is overlapped with the letter U, with the adenosine on the right and the methionine on the left. (a) Distribution of ribose zippers in the S-box RNA. Blue, interactions near the binding pocket; pink, interaction stabilizing the K-turn in P2; cyan, interaction orienting the P2 pseudoknot. Boxed interactions around the binding pocket are shown in greater detail in (b). (b) Zoom-in view of the SAM-binding pocket. The interactions are further divided into three groups, colored in slate, orange, and purple to represent their respective locations within the binding pocket. Red dashes are the important interactions involved. The backbone color scheme is the same as that in Fig. 1a and b.

Fig. 4.

Magnesium ion Ma mediates tertiary folding of the *B. subtilis yitJ* RNA. Ma ties the J3/4 U-turn and J1/2 together, sealing the SAM-binding site at the methionine tail end. The labeling and base color scheme is the same as that in Fig. 1b and c. The 3.0-Å simulated annealing omit map is contoured at 1 σ . Distances are given in angstrom in red.

Fig. 5. SHAPE mapping of conformational differences between unliganded and liganded *B. subtilis* *yitJ* riboswitches. Left panel, profile of the full-length RNA construct containing both the aptamer domain and the expression platform; right panel, *B. subtilis* *yitJ* aptamer domain only. In both panels, lane 1, dideoxy sequencing ladder revealing adenosines (runs 1 nt higher than the actual corresponding base position); lane 2, RNA+Mg²⁺; lane 3, RNA+Mg²⁺+SAM; lane 4, RNA alone (no 1M7) background. Nucleotide numbers are marked on the left and structural motifs are marked on the right. The color scheme of secondary structure elements is the same as that in Fig. 1. Prominent SHAPE reactivity differences between SAM-bound and unbound aptamer structures are highlighted by asterisks on the right.

Fig. 6. Hierarchical temperature induces unfolding of the *B. subtilis yitJ* aptamer domain as revealed by SHAPE. (a) Temperature-dependent SHAPE analysis of the *B. subtilis yitJ* aptamer domain in the presence of SAM. RNA structures are indicated on the right. Lane 1, dideoxy sequencing ladder revealing adenosine positions. Temperatures are indicated on top. (b) Quantified unfolding profiles grouped by domains. Profiles were computed by quantifying the intensities shown in (a) by fragment analysis and processing by ShapeFinder program.⁴⁵ Data are plotted on a linear scale; continuous blue line indicates average behavior. Nucleotides at pseudoknot, J3/4, and P1+P4 were fit to a unimolecular transition equation.

Table 1Mutational analysis of the *B. subtilis* *yitJ* S-box riboswitch

<i>yitJ</i> construct	Transcription termination (%) ^a		SAM binding (K_d) (μ M)
	–SAM	+SAM	
Wild-type	29	99	0.019
G11A	49	57	ND ^b
G11C	81	83	ND
G11U	70	70	ND
G79A	89	90	ND
G79C	90	90	ND
G79U	89	88	ND
C45A	90	95	ND
C45G	84	83	ND
C45U	99	99	ND
A46C	92	100	ND
A46G	82	84	ND
A46U	90	99	ND
U78A	87	96	
U78C	81	92	
U78G	89	89	ND
A47C	82	90	
A47G	71	98	
A47U	82	97	
C48A	62	100	
C48G	75	96	
C48U	85	100	
G77A	85	100	
G77C	72	94	
G77U	83	83	
U85A	46	91	
U85C	42	93	4.2
A109C	19	98	
A109G	19	92	2.1
U85C/A109G	27	99	1.1
A111G	15	43	4.2
U7G/A111U	12	68	0.56
U7G/A111C	88	97	0.41

^aEfficiency of transcription termination *in vitro* in the absence (–SAM) or presence (+SAM) of SAM (140 μ M).^bND, not detectable (K_d > 200 μ M).

Table 2

Data collection and refinement statistics

Data collection	CHESS F1
Space group	$P3_121$
Unit cell parameters	
a, b, c (Å)	$a=58.732, b=58.732, c=204.091$
α, β, γ (°)	$\alpha=90, \beta=90, \gamma=120$
Wavelength (Å)	0.9181
Resolution (Å)	20–3.0
Redundancy [overall (outer shell)]	4.0 (2.5)
Average $I/\sigma(I)$	8.0 (1.9)
Reflections	8152 (931)
Completeness (%)	94.4 (89.4)
R_{sym}^a	0.12 (0.43)
Refinement	
Resolution (Å)	20–3.0
No. of reflections used in the working set	7350
No. of reflections used in the testing set	802
Average B -factor (Å ²)	72.4
R_{work} (%) ^b	23.6
R_{free} (%) ^b	28.6
r.m.s.d. bond lengths (Å)	0.007
r.m.s.d. bond angles (°)	1.57

^a $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the statistically weighted absolute intensity of multiple measurements of symmetry-related reflections. ‡‡§

^b $R = \sum |F_o - k|F_c| / \sum |F_o|$, R_{work} from the working set and R_{free} from the test set.