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Structural analysis of a class III preQ₁ riboswitch reveals an aptamer distant from a ribosome-binding site regulated by fast dynamics


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PreQ₁-III riboswitches are newly identified RNA elements that control bacterial genes in response to preQ₁ (7-aminomethyl-7-deazaguanine), a precursor to the essential hypermodified tRNA base queuosine. Although numerous riboswitches fold as H-type or HL₄out-type pseudoknots that integrate ligand-binding and regulatory sequences within a single folded domain, the preQ₁-III riboswitch aptamer forms a HL₄out-type pseudoknot that does not appear to incorporate its ribosome-binding site (RBS). To understand how this unusual organization confers function, we determined the crystal structure of the class III preQ₁ riboswitch from Faecalibacterium prausnitzii at 2.75 Å resolution. PreQ₁ binds tightly (Kₘ,app 6.5 ± 0.5 mM) between helices P₁ and P₂ of a three-way helical junction wherein the third helix, P₄, projects orthogonally from the ligand-binding pocket, exposing its stem-loop to base pair with the 3′ RBS. Biochemical analysis, computational modeling, and single-molecule FRET imaging demonstrated that preQ₁ enhances P₄ reorientation toward P₁-P₂, promoting a partially nested, H-type pseudoknot in which the RBS undergoes rapid docking (k_dock ~0.6 s⁻¹) and undocking (k_undock ~1.1 s⁻¹). Discovery of such dynamic conformational switching provides insight into how a riboswitch with bipartite architecture uses dynamics to modulate expression platform accessibility, thus expanding the known repertoire of gene control strategies used by regulatory RNAs.

Significance

Riboswitches are RNA molecules found mostly in bacteria that control genes by sensing cellular levels of metabolites, such as the simple organic compound preQ₁. The diversity of riboswitches and their potential as novel antibiotic targets continue to elicit interest in these regulatory sequences. Here we present the crystal structure of a newly discovered bacterial preQ₁-III riboswitch that senses preQ₁ using an unusual, two-part architecture. A complementary analysis of flexibility and dynamics showed that recognition of preQ₁ induces riboswitch compaction, while concomitantly enhancing formation of a distant double-helix possessing a regulatory signal that zips and unzips rapidly, producing gene “off” and “on” states. These observations expand our knowledge of riboswitch construction and suggest a broader role for dynamics than previously recognized.


The authors declare no conflict of interest.

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4R2D).

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confined to an atypically organized HL$_{out}$-type pseudoknot that does not appear to incorporate its downstream expression platform. Biochemical analysis has not identified the location or mode of preQ$_1$ binding, and the backbone flexibility of both RBS and anti-RBS sequences did not modulate appreciably as a function of preQ$_1$ concentration (23), unlike class I and II preQ$_1$ riboswitches that show clear preQ$_1$-dependent RBS sequestration (16, 18). Crystals of the 101-mer were grown from organic salts at neutral pH and showed similar 1:1 binding stoichiometry (11, 18). Crystals of the 101-mer were grown from organic salts at neutral pH and showed similar 1:1 binding stoichiometry (11, 18). Crystals of the 101-mer were grown from organic salts at neutral pH and showed similar 1:1 binding stoichiometry (11, 18). Crystals of the 101-mer were grown from organic salts at neutral pH and showed similar 1:1 binding stoichiometry (11, 18).

To elucidate the molecular basis for ligand recognition and translational regulation by the class III preQ$_1$ riboswitch, we determined the crystal structure of the intact sensing domain from Faecalibacterium prausnitzii in complex with preQ$_1$ at 2.75 Å resolution. We used isothermal titration calorimetry (ITC), chemical modification (selective 2′-hydroxyl acylation analyzed by primer extension, or SHAPE), computational modeling, and single-molecule FRET (smFRET) analyses to relate the atomic-level details of ligand binding to conformational dynamics. Our results show how preQ$_1$ binding within an atypically organized HL$_{out}$-type pseudoknot can promote a globally compact fold. This conformation increases the population of molecules competent to form a second downstream pseudoknot, wherein the RBS docks dynamically within a helix distal to the aptamer domain. The discovery of such rapidly interconverting conformational states broadens our understanding of regulatory RNA structure, and supports a new role for dynamics in riboswitch-mediated control of protein translation.

Results

Ligand Binding and Bipartite Organization of the PreQ$_1$-III Riboswitch Structure. The F. prausnitzii riboswitch of this investigation comprises 101 nucleotides of the wild-type sequence encompassing the predicted 5′ pseudoknot and the 3′ RBS (23) (Fig. 1B). This construct binds preQ$_1$ with an apparent $K_d$ of 6.5 ± 0.5 nM and a binding stoichiometry of 1:1 (Table S1 and Fig. S1A; Methods and SI Methods). These results are comparable to values measured for class I and II family members, which produced apparent $K_d$ values of 7.3 nM and 17.9 nM, respectively, and showed similar 1:1 binding stoichiometry (11, 18). Crystals of the 101-mer were grown from organic salts at neutral pH and the phase problem was overcome by single-isomorphous replacement with anomalous scattering; the initial structure was then refined to 2.75 Å resolution to acceptable R$_{free}$ and geometry values (Table 1). The global tertiary fold is $\lambda$-shaped with dimensions of 92 × 80 × 32 Å (Fig. 1C). The aptamer is composed of contributions from four pairing regions, P1–P4 (Fig. 1B and C), organized as a HL$_{out}$-type pseudoknot wherein P3 and P4 reside in extended loop, L3, located in the 3′ tail (Fig. S2A). P1 is longer than predicted (23) because consecutive purines form Watson–Crick pairs that extend the helix (Fig. 1B and C). This conserved feature allows P1 insertion between P2 and P3, forming a coaxial stack that becomes continuous when preQ$_1$ binds in the pocket formed by the P1–P2–P4 helical junction (Fig. 1B and C). The stacked P1–P3 feature (Fig. 1D) clarifies why mutations that disrupted the proximal end of P3 led to reduced ligand affinity, whereas distal mutations within this helix showed only nominal effects (23). P4 projects orthogonally from the P2–P1–P3 coaxial stack, forming a 43-Å-long helix whose stem-loop is complementary in sequence to the 3′ terminus of the riboswitch (Fig. 1B and C). However, rather than engaging in the 7-bp helix predicted by bioinformatics (23), the
extended 3′ tail of the HL22-base pseudoknot and provide base stacking interactions that buttress the binding-pocket floor and ligand, respectively (Fig. 2A and B). With regard to the floor, prior bioinformatic and biochemical analyses predicted P2 formation with caution due to its lack of covariation and A-U richness (23). These attributes are explained by the structure because bases U8 through A10 of J1-2 form major-groove triplexes with the Hoogsteen edges of bases A85–A87, stabilizing the P2 helix while fortifying the pocket floor (Fig. 2C). The identification of consecutive UAU-AU triples in the structure also explains why prior U14–A87 and U15–A86 transversion mutations lowered preQ1 affinity (23). Overall, the quadruple triplex motif provides a stable, flat surface to recognize the mostly planar preQ1 ligand, whose presence is integral to the formation of a stable P2–P1–P3 coaxial stack (Fig. 1B and C and Fig. S2A).

**Base Triples of the PreQ1-III Aptamer Exhibit Similarities to the Ligand-Recognition Motifs of Other Riboswitches.** Use of major-groove base triples for effector recognition is a molecular motif that the preQ1-III riboswitch shares with a handful of other regulatory RNAs. The preQ1-II and preQ1-III riboswitches recognize preQ1 by using a common constellation of bases and underlying UAU-AU triples that superimpose with an rmsd of 1.1 Å (Fig. 3A). A notable difference in this conformation is that the hydrogen-bonding pattern between hydrogen-bonding partners of the preQ1 and the Watson–Crick face of C8 in the preQ1-II riboswitch is consistent with bifurcation (12). This mode of imine hydrogen bonding by the ligand is not evident in the preQ1-III structure, and appears to be a source of positional differences in the respective structures (Fig. 3A). Interestingly, the SAM-II riboswitch uses a similar array of major-groove triples to recognize the adenine moiety of SAM (8) (Fig. 3B); these nucleotides superimpose on the triplexes of the preQ1-III riboswitch with an rmsd of 0.82 Å. SAM overlaps U17 of the preQ1-III riboswitch in the C7-preQ1•U17 triplet, whereas preQ1 overlaps U44 of the U16•U44•SAM triplet. Beyond variations in the ligand-recognition triple, the underlying UAU-AU triples of the preQ1-II, preQ1-III, and SAM-II riboswitches show substantial spatial similarity (Fig. 3A and B). The cyclic-di-guanosine-monophosphate-II (c-di-GMP-II) riboswitch also uses major-groove triples to bind the guanine bases of its ligand (34), and produces a local superposition of 2.0 Å compared with the preQ1-III riboswitch (Fig. 3C). This degree of structural homology is noteworthy given the fact that these riboswitches do not share any common bases in this region. In light of the distinct evolutionary origins and diverse tertiary folds of these riboswitches examined here, this analysis highlights the resilience and versatility of triplexes in the recognition of nucleobase ligands, which should facilitate prediction of regulatory RNA function based on sequence.

**Thermodynamic Analysis of PreQ1 Binding-Site Mutants Supports the Observed Mode of Ligand Recognition.** To evaluate the thermodynamics of folding, divalent ion requirements, and to validate the structural basis of preQ1 recognition by the preQ1-III riboswitch, we conducted a series of binding experiments in solution using ITC. Effector binding by the preQ1-III riboswitch is enthalpy driven with a ΔH of −26.8 ± 0.2 kcal mol−1, which more than offsets the unfavorable entropy of 15.8 ± 0.2 kcal mol−1 (Table S1). Prior in-line probing experiments on the wild-type Ppr riboswitch sequence used in this investigation (23), as well as ITC analysis on a second, minimal preQ1-III riboswitch, env 74 (Fig. S4A), indicated that the 3′ tail of the riboswitch is dispensable past P2 for preQ1 binding (Table S1 and Fig. S1B and C); these results agree with the crystal structure wherein the 3′ terminus is not involved in aptamer formation. Site-bound divalent metal ions were not observed in the preQ1-III crystal structure, and the riboswitch binds preQ1 in the absence of Mg2+, albeit with a reduction of affinity by a factor of three, and a substoichiometric

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<th>Table 1. PreQ1-III X-ray diffraction and refinement statistics</th>
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*Deterministic and non-deterministic R-merge = \[ \sum_{hkl} \left( \frac{1}{N-hkl} \sum_{i} \left| I_{i}(hkl) - \langle I(hkl) \rangle \right| \right] / \left( \sum_{hkl} \sum_{i} I_{i}(hkl) \right) \], where N is the redundancy of the data and \( \langle I(hkl) \rangle \) is the average intensity (49).
Fig. 2. Details of the preQ₁-binding pocket within a three-helix junction. (A) Close-up view of the P1–P2–P4 helical junction that binds preQ₁. The “ceiling” exhibits tandem purine base pairs emanating from P1. PreQ₁ resides in the center of a base triple-flanked by C7 and U17. Stacked bases A84 and A52 make respective N1-imino to 2′-hydroxyl group interactions with U17 and A85; the latter base stacks below preQ₁ as part of the U-A-U triplex that composes the pocket “floor.” (B) Close-up view of the preQ₁-binding site depicting the final refined ligand bathed in unbiased (average kicked) mFᵣ–DF, electron density at the 3.0 σ level. Ligand-specific readout by C7 and U17 is shown in the context of the U8+A85 Hoogsteen pair that forms the floor. The A85 phosphate group and O2 keto of U8 make complementary interactions to the 7-aminomethyl moiety of preQ₁, providing additional specificity. ΔΔG (kcal mol⁻¹) values relative to wild type are shown for various mutations tested for ligand binding (Table S1). The view is rotated ~180° about the y axis relative to Fig. 1C. (C) Major-groove base-triple pairing of J1-2 with P2 under the preQ₁-binding pocket; tandem U-A-U triplexes are flanked by a single A10•A87-U14 triplex.

A value of 0.64 (Table S1 and Fig. S1D). These observations imply that divalent ions are important for proper preQ₁-III riboswitch folding, which could be the underlying cause of reduced ligand recognition. We then analyzed various mutants of specific nucleobases observed in the structure to be important for preQ₁ binding. C7U yielded a ΔΔG of 1.5 kcal mol⁻¹ compared with wild type, suggesting one or two lost hydrogen bonds in accord with the structure (Fig. 2B, Fig. S1E, and Table S1). U17C produced a ΔΔG of 3.3 kcal mol⁻¹ (Fig. S1F and Table S1), implying two or three lost hydrogen bonds to preQ₁, which also concur with the structure (Fig. 2B). The inclined A-minor bases A52 and A84 do not hydrogen bond directly to preQ₁ and, accordingly, the respective A-to-G mutations showed smaller ΔΔG values of ~0.3 kcal mol⁻¹ and 0.8 kcal mol⁻¹ (Fig. 2B, Table S1, and Fig. S1 G and H). We hypothesize that such free-energy changes are the result of interactions gained and lost. A52G adds an exocyclic amine on its sugar edge that likely serves as a hydrogen bond donor to both the N3 and the 2′-OH of A85 in the binding-pocket floor. These favorable contacts would form at the expense of the wild-type A-minor interaction while offsetting suboptimal base stacking against the binding pocket, consistent with the modestly favorable ΔΔG of ~0.3 kcal mol⁻¹. By contrast, the A84G mutant adds a bulky N2 amine that likely forms a hydrogen bond with the 2′-OH of U17, albeit at the expense of the wild-type A-minor interaction. Beyond this compensatory interaction, the net unfavorable ΔΔG (i.e., 0.8 kcal mol⁻¹) could be the result of suboptimal π stacking with the pyrrole ring of the ligand. Overall, these findings have implications for the means by which ligand binding in the aptamer predisposes the expression platform to adopt gene regulatory conformations (discussed below).

SHAPE Reactivity Changes Are Confined to the Core of the PreQ₁-III Aptamer. To explore how preQ₁ binding influences RNA backbone flexibility in solution, we performed SHAPE analysis on the wild-type Fpr preQ₁-III riboswitch in the context of a sequencing cassette (Fig. S4B). The addition of preQ₁ to the riboswitch reduced reactivity at several positions, including P1, the U-rich region of P2, J3-4, and positions 84 and 86 (Fig. 4 A and B). Differential SHAPE-reactivity analysis showed strong P1 and P2 modulation (Fig. 4C), consistent with the crystal structure in which preQ₁ mediates coaxial stacking between these helices (Figs. 1C and 2A). Nucleotides that contact preQ₁ in the three-way junction also showed modulation, including C7 of J1-2, which recognizes the effector, and U8 of the major-groove triplex located in the floor of the binding pocket (Fig. 2A); U17 of J2-1, which directly recognizes the preQ₁ edge (Fig. 2B); A52 of J3-4, which contributes an inclined A-minor interaction to the binding pocket on one face, and base stacking upon A53 with the other (Fig. 2A and B); and A84 of J4-2, which contributes a second inclined A-minor interaction that directly abuts preQ₁.

Fig. 3. Comparison of the preQ₁-III riboswitch to other regulatory RNAs that use triplexes to recognize nucleobase ligands. (A) Overlay of the preQ₁-III riboswitch base triples (gold) upon the preQ₁-III riboswitch (deep purple; PDB ID code 2MYR) (12). The superposition is based on the major-groove base triples and preQ₁, which yielded an average rmsd of 1.1 Å. Here and elsewhere, the preQ₁-III riboswitch ligand is green, and the superimposed ligand is magenta. (B) Overlay of the preQ₁-III riboswitch base triples upon those of the SAM-II riboswitch (deep purple; PDB ID code 2QWV) (8). The superposition is based on shared major-groove base triplex nucleotides (excluding ligand), which yielded an average rmsd of 0.82 Å. (C) Overlay of the preQ₁-III riboswitch base triples with those of the c-di-GMP-II riboswitch (deep purple; PDB ID code 3Q3Z) (34). The superposition is based on shared nucleotide atoms (excluding ligand), which yielded an average rmsd of 2.0 Å.
Computational Modeling Demonstrates the Structural Feasibility of RBS Sequestration. The preQ₁-I and preQ₁-II riboswitches fold as pseudoknots that partially or fully integrate their expression platforms into the three-way helical junction that composes the effector-binding site (Fig. 4D), suggesting that preQ₁ reduces core backbone flexibility while promoting the HLo-out-type pseudoknot fold. By contrast, P3 and P4 are largely unaffected by preQ₁, implying that they are prefolded. Perhaps the most surprising observation was the apparent lack of preQ₁-dependent modulation within the anti-RBS and RBS sequences (i.e., helix P₅; Fig. 4 A and C). This result prompted us to explore the feasibility of P₅ helix formation by use of computational approaches.

Single-Molecule FRET Supports Dynamic RBS Sequestration upon preQ₁ Binding. To understand how ligand binding promotes RBS sequestration, we conducted smFRET analysis on a preQ₁-III riboswitch construct harboring fluorophores that report on helix P₅ formation (Fig. 6A and Fig. S5A). In buffer containing Mg²⁺ at a near-physiological concentration, ~90% of riboswitches showed single-step photobleaching, consistent with the majority of molecules being monomeric under the low-concentration
conditions of smFRET (Fig. 6B and Fig. S6A–C). Importantly, the monomeric state is compatible with expectations for folding and gene regulation in an intracellular environment, which is reflected by our computational model (Fig. 5). The FRET histogram of the monomeric population in the absence of preQ1 showed a two-state distribution with a major (92%) ∼0.55 mid-FRET state, and a minor (8%) ∼0.89 high-FRET state (Fig. 6C and Fig. S7A–C). Under these conditions, a large fraction of the smFRET traces remained static in the mid-FRET state before photobleaching, but a smaller fraction was dynamic with multiple transitions between the two states (Fig. 6B, Top, and Fig. S6D). In a transition occupancy density plot (TODP), ∼32% of all molecules showed dynamics (Fig. 6D, Top). This analysis demonstrates that the preQ1–III riboswitch samples compactly folded P5 conformations in the absence of ligand, similar to preQ1-I and other riboswitch classes (11, 35). As little as 25 nM preQ1 increased the fraction of dynamic traces to ∼61% with a further shift to ∼69% at saturating 1 μM preQ1 (Fig. 6B and D and Fig. S6B and C). Consistent with the small fraction of molecules occupying the transient high-FRET state at any given time, the addition of more ligand led to only a modestly larger contribution of the high-FRET state to the population histogram (14%; Fig. 6C). Though small, this increase in the high-FRET state was consistently reproducible in multiple experiments (Fig. S7A–C) and is supported by difference histogram analyses, which show decreases in the mid-FRET state and concomitant increases in the high-FRET population with added preQ1 (Fig. S7D–F).

We then examined the effect of blocking P5 formation in the context of the wild-type Fpr preQ1–III riboswitch using an 11-nt DNA strand complementary to the riboswitch 3′ terminus (Fig. 5C, Inset, and SI Methods). This anti-P5 competitor resulted in complete loss of the high-FRET state and rendered almost all molecules static in a ∼0.4 mid-FRET state, both in the absence and presence of 1 μM preQ1 (Fig. S8A–F). This finding further establishes that the high-FRET state corresponds to the P5 docked conformation wherein the RBS is sequestered. The lower mid-FRET value of ∼0.4 compared with the ∼0.55 value in the absence of DNA oligonucleotide suggests a small increase in

![Fig. 5](image-url)

**Fig. 5.** Model of the preQ1–III riboswitch showing intramolecular base pairing of the RBS in helix P5. (A) Cartoon diagram of a representative all-atom computational model demonstrating the feasibility of loop P4 engagement in an H-type pseudoknot that sequesters a portion of the RBS within helix P5, consistent with a “gene off” conformation. (B) Close-up view of P5 showing explicit base pairs between the anti-RBS of the P4 loop and the 3′ RBS, representing the “Docked” state. The view is rotated ∼90° about the z axis, and +45° about the x axis relative to A. (Inset) The model accounts for the proposed P5 secondary structure from bioinformatic analysis (23). Base-paired nucleotides of P5 are >93% conserved. Unpaired base 71 of J4-5 is present in only 75% of sequences as any base, and unpaired nucleotides 91 and 92 of J2-5 indicate a 75% preference for purine followed by any base in 95% of sequences.
separation between the fluorophores, as expected for a more rigid RNA–DNA duplex. Furthermore, addition of a small molecule known not to bind the preQ₁-III riboswitch (i.e., isoxanthopterin (IXP) or 2-aminopurine (2AP); Fig. S1 I and J and SI Methods) revealed little increase in either the high-
FRET state or the fraction of dynamic molecules, by contrast to 
preQ₁ (Fig. S8 G–P), thereby demonstrating a highly specific 
response that occurs only with the cognate ligand.

At the molecular level, the mean values of 0.55 and 0.89 for 
the mid- and high-FRET states correspond to distances of ~52 
and ~38 Å, respectively. The mid-FRET state is consistent with 
the distance of ~55 Å expected for an undocked P5 helix in 
which the RBS is solvent-exposed and flexible (Fig. 6I). The 
mean FRET value of this undocked state increased from 0.55 
to 0.62 upon preQ₁ addition (Fig. 6C), signifying a small compac-
tion in the presence of ligand. The high-FRET state agrees well 
with distances observed for the preQ₁-III riboswitch model in 
which P5 is docked (Fig. S5); specifically, the U77 to G101 dis-
tance that approximates the FRET pair was observed to be bi-
modal over 8 μs of MD simulations with maxima at ~37 and ~40 Å 
(Fig. S5 F and G). We also noted that in the absence of ligand, 
most traces displayed either static or dynamic behavior and 
switched rarely (<1%) between the two regimes (Fig. S8Q), 
which suggests that the two species interconvert slowly during 
our observation window (~5 min) before photobleaching. How-
ever, individual molecules were observed to switch in situ from 
the static to the dynamic regime upon preQ₁ addition (Fig. S8R), 
suggesting that ligand accelerates the transition into the dy-
namic, “active” conformation while the kinetics of dynamic 
molecules appeared unchanged. In the latter regard, the dynamic 
smFRET traces showed homogeneous kinetics that allowed us to 
calculate the rate constants of docking (k_\text{dock} = 0.59 \pm 0.03 \text{ s}^{-1}) 
and undocking (k_\text{undock} = 1.10 \pm 0.06 \text{ s}^{-1}) in the absence of 
preQ₁. Notably, the rate constants were not affected substantially 
by addition of 25 nM or 1 μM preQ₁ (Table 2). These relatively 
fast rate constants, and the diminutive size of the high-FRET pop-
ulation at equilibrium, provide a plausible explanation for the 
inability to observe preQ₁-dependent formation of the P5 helix by 
SHAPE (Fig. 4).

ITC analysis indicated that preQ₁ binds the class III ribo-
switch in the absence of Mg\(^{2+}\) (Table S1), a result that is 
corroborated by smFRET analysis in which Mg\(^{2+}\)-free experiments 
produced histograms similar to those in the presence of Mg\(^{2+}\) 
(Fig. S9A). However, the absence of Mg\(^{2+}\) resulted in hetero-
geneous kinetics wherein the weighted-mean value of k_\text{dock} 
increased, and k_\text{undock} was practically unchanged upon preQ₁ 
addition (Table 2). These results suggest that the preQ₁-III 
ribswitch transiently samples a docked P5 conformation even 
in the absence of both Mg\(^{2+}\) and preQ₁ (Fig. S9 B and C). 
Notably, even though the addition of preQ₁ affects P5 docking 
in the absence of Mg\(^{2+}\), the fraction of dynamically docking P5 
helices is unaltered (Fig. S9D). The lack of preQ₁ dependence 
and absence of Mg\(^{2+}\) diminish the likelihood that such dy-
namics are operative in gene control. Instead, our regulatory 
model is best framed within the context of near-physiological Mg\(^{2+}\) 
concentrations wherein the addition of preQ₁ does not alter docking and undocking rates (Table 2) but enhances the 
fraction of molecules competent to undergo dynamic P5 
docking (Fig. 6D), thus leading to RBS sequestration within the 
3’-terminal H-type pseudoknot (Fig. 5B).

Discussion
Here we report the crystal structure of a preQ₁-III riboswitch 
bound to its effector (Figs. 1C and 2), thereby establishing the 
fold of a new class of regulatory RNA. The riboswitch can be 
parsed structurally into an aptamer domain composed of an 
H-type pseudoknot that has co-opted major-groove base 
triples for ligand recognition (Fig. 3), and a downstream, par-
tially embedded H-type pseudoknot that sequesters the RBS 
(Fig. 5). Such structural organization is uncommon among 
riboswitches (7, 19) and has not been investigated to an appre-
ciable extent in terms of structure–function relationships (35). As 
such, we established a functional framework for the preQ₁-III 
riboswitch that entails ligand-dependent folding in the presence of Mg\(^{2+}\) to promote a compact aptamer that is conducive to 
dynamic docking and undocking of the remotely positioned RBS 
(Fig. 6D). Frequent docking of the RBS would sequester the 
expression platform, leading to ligand-dependent queT gene 
control by translational attenuation. This paradigm differs from 
other riboswitches, such as preQ₁-II and SAM-II, because these 
molecules integrate RBS sequences directly into their aptamer 
domains upon ligand binding (8, 10, 12, 16, 18). Consequently, 
RBS docking within these riboswitches is characterized by pro-
longed high-FRET dwell times in the presence of Mg\(^{2+}\) and 
ligand (>2.2 s and ~3.5 s, respectively) with timescales limited 
most likely by fluorophore photobleaching (10, 16). By contrast, 
the preQ₁-III riboswitch displays dwell times of ~0.9 s for the 
RBS-docked state, and ~1.8 s for the undocked state (Fig. S6 
D–F). This dynamic character differentiates the preQ₁-III 
ribswitch from other regulatory RNAs that appear to rely 
upon comparatively static conformational states to achieve 
RBS sequestration.

We then considered the molecular basis by which effector 
binding to the preQ₁-III aptamer leads to a larger population of 
riboswitches that dynamically sequester the remote RBS. Our 
results indicated that preQ₁ binding only marginally stabilizes 
P5 docking, which occurs entirely through an increasing fraction 
of molecules that dynamically access the high-FRET docked state, 
and is evident in our kinetic analysis wherein no substantive rate 
changes occurred in k_\text{dock} (~0.6 s\(^{-1}\)) or k_\text{undock} (~1.1 s\(^{-1}\)) under 
conditions containing Mg\(^{2+}\) and preQ₁ vs. those with Mg\(^{2+}\) and 
no ligand (Table 2). These observations are consistent with a 
ligand-dependent aptamer conformation that reorients pre-
formed helix P4 acutely relative to the P2–P1–P3 coaxial stack. 
Such positioning would predispose helix P5 to dock, thus com-
pleting the H-type pseudoknot with concomitant RBS burial.

### Table 2. PreQ₁-III riboswitch P5 kinetics based on smFRET

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<td>Fast</td>
<td>Slow</td>
<td>Weighted mean</td>
<td>Fast</td>
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<td>n/a (^\dagger)</td>
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<tr>
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<tr>
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<td>25</td>
<td>0.50 ± 0.01</td>
<td>0.06 ± 0.001</td>
<td>0.16</td>
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</table>

\(^\dagger\)The errors reported for the kinetics in 1.0 mM Mg\(^{2+}\) are SDs from three independent experiments.

\(^\dagger\)n/a, not applicable, because the rate was fit with a single exponential.

\(^\dagger\)For conditions with no Mg\(^{2+}\), the SEs are derived from the quality of fits to a double-exponential function.
Our computational model demonstrates the feasibility and stability of this relatively compact fold (Fig. 5 and Fig. S5 D and E), which requires preQ₁ binding for full efficacy (Fig. 6D and Fig. S8).

Coupling of preQ₁ binding within the H₀₄₅-type pseudoknot aptamer to distal RBS sequestration within the H₄₅₅-type pseudoknot depends on inclined A-minor base A52 of J₃-₄ and base A84 of J₄-2, which are 75% and 97% conserved (25). ITC confirmed that both adenines affect preQ₁ binding (Fig. 2B and Table S1), although neither base hydrogen bonds directly to the ligand. SHAPE analysis revealed greater backbone stability of these nucleotides in the presence of preQ₁, which is corroborated by the crystal structure (Fig. 4 C and D). Specifically, preQ₁ engages in a T-shaped π-stacking interaction with A84, which simultaneously forms a cross-strand stacking interaction with A52 (Fig. 7A). Importantly, the stacking of A84 upon A52 abuts a network of continuously stacked bases in helix P4 that begins at A53 and culminates in the anti-RBS sequence (Fig. 7A). This stacking network is maintained in the computational model wherein helix P5 is docked by pairing of the anti-RBS and RBS sequences, which requires helix P4 repositioning (Fig. 7B). In this manner, preQ₁ binding establishes a continuous stacking network that predisposes P4 to reorient acutely toward the P₁–P₂ coaxial stack, favoring P₅ docking and RBS sequestration.

Although the broader role of inclined A-minor bases in the context of H₄₅₅- and H₀₄₅₅-type pseudoknots is to stabilize a 5' terminal stem (7), the preQ₁-II riboswitch provides a precedent for the use of such adenine bases in mediating base stacking between a ligand and a nearby orthogonal stem-loop (Fig. 7C). Specifically, preQ₁ binding to the class II riboswitch alters P₄ helical dynamics and its proximity to the orthogonally oriented aptamer domain (12, 16). Like the preQ₁-III riboswitch, ITC analysis of preQ₁-II A-minor adenines verified the importance of these bases in preQ₁ binding, although larger ΔΔG values were observed upon mutagenesis indicative of greater losses in ligand binding (12). Nonetheless, there is notable structural homology between the inclined A-minor adenines of the preQ₁-II and preQ₁-III riboswitches (Fig. 7A and B vs. Fig. 7C) that lends support for the mode by which preQ₁ binding to the preQ₁-III aptamer can influence the orientation of helix P₄ in a manner that favors formation of distal helix P₅ and concurrent RBS sequestration. Conversely, ligand deficiency in the preQ₁-III aptamer domain destabilizes helices P₁ and P₂, resulting in increased flexibility of the inclined A-minor bases (Fig. 4). Thus, although a fraction of the riboswitch population can dynamically sequester the RBS in the absence of ligand, translational control by the preQ₁-III riboswitch requires a folded aptamer and preQ₁ binding for greatest efficacy (Fig. 6D and Fig. S8).

Overall, our results provide a molecular-level framework to understand how effector binding within the preQ₁-III riboswitch aptamer influences the conformation of a distal expression platform. In this context, the dynamic character of the RBS is unusual compared with other riboswitches, and our work demonstrates how a nonintegrated expression platform can achieve ligand-dependent translational attenuation without burial in the aptamer core. This paradigm is likely applicable to other riboswitches, especially those with bipartite structural organization, thus expanding the known repertoire of translational attenuation strategies.

**Methods**

**Riboswitch Production and Isothermal Titration Calorimetry.** *Faecalibacterium prausnitzii* (Fpr) preQ₁-III riboswitches and mutants thereof (Fig. 1B and Table S1) were generated by in vitro transcription and purified by denaturing PAGE (36). The 74 env sequences (Fig. S4A) were produced by chemical synthesis (GE Life Sciences) and HPLC purified (37); preQ₁ was prepared as described (18). Lyophilized RNA was suspended in a folding buffer comprising 0.050 M Na-Hepes (pH 7.0) containing 0.10 M NaCl. The Fpr RNA was heat to 65 °C for 5 min followed by addition of 0.006 M MgCl₂ or 0.0005 M EDTA before slow cooling. The 74 env RNA was folded by heating each strand at 70 °C, mixing the strands, and incubating at 37 °C for 20 min followed by flash cooling on ice. ITC measurements were conducted using a VP-ITC calorimeter (MicroCal, Inc.) as described (38) in which the folding buffer above included 0.006 M MgCl₂ or 0.0005 M EDTA to produce ITC buffer. Each sample (Fig. S1) was dialyzed at 4 °C overnight against 4 L of ITC buffer. RNA was diluted with dialysis buffer to 3.1–3.3 M for wild-type Fpr, 3.2–7.8 M for the A52G and A84G Fpr mutants, 10.5–15.7 M for the C7U and U17C Fpr mutants, 2.4–3.6 M for 74 env, and 1.8 M for 74 env–s230–43. PreQ₁ was dissolved in dialysis buffer at concentrations ~10-fold higher than RNA. Thermograms were analyzed with Origin 7.0 (MicroCal) using a 1:1 binding model. Average thermodynamic parameters and representative thermograms with curve fits are provided (Table S1 and Fig. S1). See SI Methods for details of the IXP and ZAP control experiments.

**Riboswitch Crystallization and X-ray Data Collection.** PreQ₁-III riboswitch RNA (Fig. 1B) was dissolved to 0.16 mM in 0.010 M Na-cacodylate (pH 7.0). RNA
was folded by heating to 65 °C for 3 min followed by addition of 0.006 M MgCl₂, 0.32 mM preQ₁, and 0.25 mM preQ₁. Subsequently, the RNA was heated to 65 °C for 5 min, followed by a 2,500–fold centrifugation step. Crystals were prepared by the hanging-drop vapor-diffusion method in which 1.6–2.0 μL of folded RNA was mixed with an equal volume of well solution, followed by equilibration over 1 mL of well solution at 20 °C. Crystals grew as hexagonal rods within 24 h and achieved a maximum size of 0.2 × 0.05 × 0.05 mm within a week. See SI Methods for crystallization solutions. All crystals were flame-frozen by washing in well solution supplemented with 0.32 mM preQ₁, then plunging into N₂ solution. X-ray diffraction data for phased were recorded at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 7-1. Phasing module (PM) data were reduced with HKL2000 software (39). High-resolution data were recorded at SSRL beamline 11-1 and reduced with XDS/SCALE (40) (Table 1).

**Phase Determination, Refinement, and Analysis.** Experimental phases were obtained by single isomorphous replacement with anomalous scattering (SIRAS) to 3.0 Å resolution. Subsequently, the resolution was extended to 2.75 Å resolution with refinement to reasonable R(fact) and geometry (Table 1). Details are available in SI Methods.

**Chemical Modification by Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension.** The ligand-dependent acylation of the Fpr preQ₁-III riboswitch was probed by primer extension (43, 44). 3′-PHC (5′-AAG GTT GTA TGA TGC GAA CTA TCT-3′) was synthesized as described (42). Purified RNA from in vitro transcription (36) was heated in metal-free water for 2 min at 95 °C, then flash-cooled on ice. A 3× SHAPE buffer (0.333 M Hepes (pH 8.0), 0.02 M MgCl₂, 0.333 M NaCl) was added and the RNA was equilibrated at 37 °C for 10 min. A total of 1 μL of preQ₁ stock (1 M in 1× PBS) was added to the RNA. The RNA was incubated for 15 min. To this mixture, 1 μL of 10× NAI stock in DMSO (+) or DMSO alone (−) (Fig. 4 A and B), was added to a final concentration of 0.06 M. The NAI reaction proceeded for 15 min followed by one extraction with acid phenol:chloroform (pH 4.5 ± 0.2) and two with chloroform. RNA was precipitated with 40 μL of 3 M sodium acetate (pH 5.2) containing 1 μL of glycercol (20 μg μL⁻¹). Pellets were washed twice with 70% ethanol and suspended in 10 μL RNase-free water. Extension using 32P-labeled primer and data analysis were as described (41, 42).

**Riboswitch Modeling and MD Simulations.** To evaluate the feasibility of PS base pairing, a steered MD simulation was performed starting from the Fpr crystal structure (Fig. 1C). The P4 loop (nucleotides 64–70) was moved toward nucleotides 93–99 using distance restraints with three force-constant steps applied over the total time of 10 ns. The equilibrium distance between the heavy atom of the hydrogen bond acceptor to the hydrogen atom of the donor was set to 2.5 Å. First, the distance restraint force constant was ramped from 0 to 5 kcal (mol Å⁻²)⁻¹ in 100 ps, followed by an interval of 500 ps, followed by slow cooling and 0.2-μm filtration. Crystals were prepared by the hanging-drop vapor-diffusion method in which 1.6–2.0 μL of folded RNA was mixed with an equal volume of well solution, followed by equilibration over 1 mL of well solution at 20 °C. Crystals grew as hexagonal rods within 24 h and achieved a maximum size of 0.2 × 0.05 × 0.05 mm within a week. See SI Methods for crystallization solutions. All crystals were flame-frozen by washing in well solution supplemented with 0.32 mM preQ₁, then plunging into N₂ solution. X-ray diffraction data for phased were recorded at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 7-1. Phasing module (PM) data were reduced with HKL2000 software (39). High-resolution data were recorded at SSRL beamline 11-1 and reduced with XDS/SCALE (40) (Table 1).

**Single-Molecule FRET Analysis.** The Fpr preQ₁-III riboswitch was produced by chemical synthesis (GE Life Sciences) from two RNA strands (Fig. 4C). The feasibility of producing a functional split riboswitch was demonstrated by ITC analysis of the 74 env preQ₁-III riboswitch, which yielded an apparent Kd of 10.1 ± 2.5 nm (Table S1 and Figs. S1 and S4A), indicating high-affinity preQ₁ binding. Extension of the RNA smFRET strand allowed hybridization to a biotinylated DNA tether (IDT, Inc.). The RNA strand harboring the RBS included 5-amino-allyl-U77 and Dy547 at the 3′ terminus. A Cy5 label (GE Healthcare) was added as described (46), and both RNA strands were PAGE-purified and desalted (36). To fold the RNA, 1 μM of each strand was combined and annealed at 70 °C for 3 min in 0.05 M Hepes–KOH (pH 7.0). KCl was added to a concentration of 0.1 M with additional heating at 70 °C for 2 min followed by 5-min incubation at 37 °C. The RNA was cooled to 23 °C for 10 min. smFRET experiments were performed using a prism-based total internal reflection fluorescence (TIRF) microscope (Fig. 6A) (11, 47). Briefly, quartz slides containing microfluidic channels were coated with biotinylated BSA followed by streptavidin treatment. Unbound protein was washed away with 1× smFRET buffer (0.050 M Hepes–KOH (pH 7.0), 0.1 M KCl, or with without 0.001 M MgCl₂). Immobilization of 10–25 pM of folded riboswitch was achieved using the biotin–streptavidin interaction. Unbound molecules were washed away with 1× smFRET buffer. An oxygen scavenging system (5S35) containing 5 mM protocatechuic acid, 50 mM protocatechuic acid 3,4-dihydroxylase, and 2 mM Trolvox in smFRET buffer was used to prolong fluorophore longevity and reduce photoblebbing (11). Molecules were imaged by an intensified-CCD camera (i-PentaMAX; Princeton Instruments) at a time resolution of ~60 ms in the absence or presence of various preQ₁ concentrations. Dy547 was excited directly using a 532-nm laser, and emission intensities from both Dy547 (donor, I₅) and Cy5 (acceptor, I₆) were recorded simultaneously, and used to calculate the FRET ratio as I₅/I₆ + I₅ after background correction. The raw FRET movies were processed using IDL (Research Systems) to extract time traces of individual molecules, and analyzed further by custom MATLAB (MathWorks) scripts. See SI Methods for further details.

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