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Structural insights into amino acid binding and gene control by a lysine riboswitch

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Abstract

In bacteria, the intracellular concentration of several amino acids is controlled by riboswitches^{1–4}. One of the important regulatory circuits involves lysine-specific riboswitches, which direct the biosynthesis and transport of lysine and precursors common for lysine and other amino acids^{1–3}. To understand the molecular basis of amino acid recognition by riboswitches, here we present the crystal structure of the 174-nucleotide sensing domain of the *Thermotoga maritima* lysine riboswitch in the lysine-bound (1.9 Å) and free (3.1 Å) states. The riboswitch features an unusual and intricate architecture, involving three-helical and two-helical bundles connected by a compact five-helical junction and stabilized by various long-range tertiary interactions. Lysine interacts with the junctional core of the riboswitch and is specifically recognized through shape-complementarity within the elongated binding pocket and through several direct and K⁺-mediated hydrogen bonds to its charged ends. Our structural and biochemical studies indicate preformation of the riboswitch scaffold and identify conformational changes associated with the formation of a stable lysine-bound state, which prevents alternative folding of the riboswitch and facilitates formation of downstream regulatory elements. We have also determined several structures of the riboswitch bound to different lysine analogues⁵, including antibiotics, in an effort to understand the ligand-binding capabilities of the lysine riboswitch and understand the nature of antibiotic resistance. Our results provide insights into a mechanism of lysine-riboswitch-dependent gene control at the molecular level, thereby contributing to continuing efforts at exploration of the pharmaceutical and biotechnological potential of riboswitches.

RNA sensors play a crucial part in many regulatory loops, owing to their capacity for directing gene expression in response to various stimuli in the absence of protein participation^{6–8}. Recent three-dimensional structures of a thermosensor⁹, a metallosensor¹⁰, a metabolite-bound ribozyme^{11,12} and riboswitches specific for purine nucleobases^{13,14}, and for co-enzymes thiamine pyrophosphate^{15–17} and S-adenosylmethionine^{18,19} have highlighted how each ribosensor uses unique structural features to sense its cognate stimulus. However, the molecular details of the organization of amino-acid-specific

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Author Contributions L.H. crystallized the *T. maritima* lysine riboswitch; A.S. determined the structures and was assisted by L.H. during refinement; A.S. and L.H. performed biochemical experiments; and A.S. and D.J.P. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information Atomic coordinates of the X-ray structures of the lysine riboswitch bound to ligands have been deposited in the RCSB Protein Data Bank under the following accession codes: lysine, 3DIL; AEC, 3DIG; L-4-oxalysine, 3DJ0; homoarginine, 3DIQ; and iminoethyl-L-lysine, 3DIR. Codes for other structures are: free state, 3DIS; [Ir(NH₃)₆]³⁺-soaked, 3DIO; Cs⁺-soaked, 3DIM; TI⁺-soaked, 3DJ2; Mn²⁺-soaked, 3DIY; K⁺-anomalous, 3DIX; and Mg²⁺-free form, 3DIZ.

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riboswitches, such as the lysine riboswitch, which efficiently discriminates against other free amino acids, their precursors and amino acids within a peptide context^{1,2,5}, remain obscure. The determination of the lysine riboswitch structure presents a considerable challenge, because of its large metabolite-sensing domain, predicted to form a five-way junction¹⁻³.

The *T. maritima* riboswitch is a typical lysine riboswitch^{1-3,5} (Supplementary Figs 1 and 2a) which uses a transcriptional attenuation mechanism to repress the production of aspartate-semialdehyde dehydrogenase³, which is involved in the synthesis of a precursor for methionine, threonine, lysine and diaminopimelate. The structure of the lysine-bound riboswitch domain, also known as an 'L box', features three-helical and two-helical bundles radiating from a compact five-helical junction (Fig. 1a-c and Supplementary Fig. 2b-d) that contains lysine inserted into its core. The junction is organized on the basis of a modified four-way junction²⁰ through colinear stacking of helices P1 and P2, and helices P4 and P5, positioned as two intersecting lines of an uneven letter 'X'. The P2-P2a-L2 stem-loop reverses its orientation through two turns important for riboswitch function^{5,21}: one of them adjacent to a loop E motif^{22,23} and the other centred on a turn that replaces the kink-turn motif²⁴ found in other lysine riboswitches (Supplementary Fig. 3). Stems P2 and P3 are aligned by an unusual kissing-loop complex between loops L2 and L3 (Fig. 1d and Supplementary Fig. 4), whereas parallel stems P2 and P4 are anchored by a conserved loop (L4)-helix (P2) interaction (Fig. 1e).

The five-helical junction contains three layers of nucleotides, each composed of two interacting base pairs, organized around the centrally positioned lysine which fits into a tight pocket and is specifically recognized by its charged ends (Fig. 2a-c). This lysine-bound pocket architecture (Fig. 2) is also retained in lysine analogue complexes (Fig. 3) outlined later. The top layer, composed of G14-C78 and G115-C139 base pairs (Fig. 2d), is stabilized through ribose zipper and type I A-minor triple interactions with invariant A81, which in turn is paired with Na⁺-bound G80 (Fig. 2d and Supplementary Fig. 5a). The middle layer, containing G12-C79 and G114-U140 base pairs, forms specific interactions with the bound lysine (Fig. 2e). Both the carboxylate and ammonium groups of the lysine 'main chain' segment are hydrogen-bonded to the minor groove edges of purine bases and sugar 2'-OH groups (Fig. 2b, e), whereas the ϵ -ammonium protons of the side chain form hydrogen bonds with a non-bridging phosphate oxygen, a sugar ring oxygen and a tightly bound water molecule, W1 (Fig. 2c). In addition, lysine is sandwiched between the A81 base (Fig. 2d) and the G11-G163 base pair from the bottom junctional layer (Fig. 2e and Supplementary Fig. 5b), thereby stabilizing the top of the P1 helix which is necessary for gene expression control. The bound lysine facilitates the holding together of the stacked P1-P2 and P4-P5 helical segments, and contributes to the positioning of the P3 helix by locking up G80, which is placed over the ϵ -ammonium group of lysine, and stacks with the A82-U113 base pair of P3 (Fig. 2a). This stable junctional conformation is further reinforced by a tertiary stacking interaction between G110 and A164 (Fig. 2a) characteristic for riboswitches from thermophiles, as well as other interhelical contacts (Figs 1b, c and 2a). A notable feature of the lysine-binding pocket is a K⁺ cation (Fig. 2b), which binds a carboxyl oxygen of lysine and zippers up the junction using several coordination bonds. The K⁺ cation is directly observed on the anomalous map (Supplementary Fig. 6) and is replaceable by its mimics, Cs⁺ and Tl⁺, but not by Mn²⁺ (Supplementary Figs 7-9), a mimic of Mg²⁺ (refs 25 and 26). The importance of K⁺ for lysine binding has been demonstrated in primer extension experiments. In the presence of lysine and at physiological concentration of K⁺, reverse transcriptase pauses before the junction at A169, reflecting the formation of a stable junctional conformation (Fig. 4a, lanes 8, 10). The pause decreases 33-fold after the replacement of K⁺ by Na⁺ (Fig. 4a, lanes 2, 4), suggesting that there is either reduced stability or failure to generate a junction competent for lysine recognition under K⁺-free conditions. These results have been supported by equilibrium dialysis experiments with *T.*

maritima and *Bacillus subtilis* riboswitches. In both cases, lysine binding affinity significantly decreased when K^+ was omitted or replaced by Na^+ (Fig. 4b). Note that lysine binds better to a riboswitch from a thermophile than from a mesophile. The preference of K^+ for binding to the negatively charged carboxylate group contrasts with Mg^{2+} -mediated phosphate recognition in other ribosensors^{11,12,15,16} and might also be a characteristic for other amino-acid-specific riboswitches. Because more than 20 lysine-binding proteins (listed in the Protein Data Bank) do not use cations to mediate lysine recognition, this feature is probably unique to RNA, given that it lacks the positively-charged side chains found in proteins. Folding of most RNAs, including the *B. subtilis* lysine riboswitch²¹, requires Mg^{2+} . However, the crystals of the *T. maritima* lysine riboswitch can be grown in the absence of Mg^{2+} (Supplementary Fig. 10). Moreover, a Mn^{2+} soak does not replace cations in the structure grown with Mg^{2+} , and, on the basis of the coordination distances, most of these cations can be assigned as Na^+ . These results indicate that the L box from a thermophile does not critically depend on Mg^{2+} , the function of which can be co-opted by monovalent cations. The identification of antibiotic-resistance mutations^{27,28} in the lysine riboswitch^{1,2}, together with the demonstration of a direct interaction between the riboswitch and lysine-like antibacterial compounds^{1,2,5}, suggest that riboswitch targeting, along with other processes²⁹, is an important component of the antibiotic activity. To understand the molecular basis of antibiotic resistance and explore the pharmaceutical potential of the lysine riboswitch, we have determined structures of the riboswitch bound to antibacterial compounds S-(2-aminoethyl)-L-cysteine (AEC) and L-4-oxalysine⁵, which contain sulphur and oxygen at position C4, respectively (Fig. 3a). Because the pocket has a small cavity between the C4 and N7 positions of bound lysine (Fig. 3c and Supplementary Fig. 11a), both C4-substituted analogues can be placed within the pocket in a manner similar to bound lysine (Fig. 3b, top panel), suggesting the potential for incorporation of even larger C4-substituents. Despite similar placement within the pocket, primer extension assays suggest that there is weaker binding of AEC and L-4-oxalysine to the riboswitch (Fig. 4c and Supplementary Fig. 12), possibly due to an increased electronegativity of substituents at the C4 position. Next, we determined the structures with lysine analogues L-homoarginine and N6-1-iminoethyl-L-lysine, where the ϵ -ammonium group of lysine is replaced by a guanidinium group and its methyl-substituted variant, respectively (Fig. 3a)⁵. In these structures (Fig. 3b), the side chains of lysine analogues are slightly shifted to provide better stacking interactions with the G80 base. The nitrogen atoms of the guanidinium-like extensions replace water molecules W1 and W2, found in the lysine complex (Fig. 2b). The G163 sugar is slightly rotated, so that the hydrogen bond pattern of W1 is retained by both ligands, whereas L-homoarginine forms extra hydrogen bonds with G163. Although most RNA-ligand contacts are preserved, both analogues demonstrate weaker interactions than lysine in primer extension (Fig. 4c and Supplementary Fig. 12) and in-line probing⁵ experiments, emphasizing the fine structural complementarity between the ϵ -ammonium group of lysine and RNA. The lysine-binding pocket has two openings, which could be exploited for the design of next generation lysine-like analogues (Fig. 3c, d and Supplementary Fig. 11b, c). One of the openings could accommodate modifications or extensions of the carboxylate group, possibly by substituting the K^+ cation. The other smaller opening could allow extensions from N9 of L-homoarginine and iminoethyl-L-lysine. The analogue-bound structures and the biochemical data⁵ indicate that the lysine-binding pocket is rather rigid, and only accommodates compounds which can sterically fit the pocket. Therefore, lysines in a polypeptide chain and branched amino acids are not recognized by the riboswitch. The lysine riboswitch also discriminates against smaller amino acids that fit into the pocket (data not shown) but are unable to make essential intermolecular contacts in the vicinity of G80. To gain insights into lysine-induced conformational rearrangements of the riboswitch in solution, we performed footprinting experiments using in-line probing, specific for flexible RNA regions, and cleavage by the nucleases T2 (single-stranded RNA) and V1 (paired and stacked regions). As in *B.*

subtilis^{1,5}, the transition from the free to the lysine-bound states of the *T. maritima* riboswitch is accompanied by conformational changes within the junctional core, detected as strong cleavage reductions in both in-line and V1 probing (Fig. 4d–f and Supplementary Fig. 13). However, the kissing loop complex is probably preformed in the free riboswitch^{5,21}, as evident from the absence of cleavages in in-line^{1,5} (Fig. 4d) and T2 probing (Supplementary Fig. 13b), coupled with V1 cleavages at nucleotides 45–46 (Fig. 4e), which are only weakly enhanced by lysine binding. The P2–L4 tertiary contact seems to be more dynamic in character as reflected in the rather strong T2 (nucleotides 126–127, Fig. 4f) cleavage patterns in the lysine-free form, coupled with only weak protection on complex formation. The projection of the in-line cleavage reductions (this study and ref. 1) on the structure (Fig. 4g) provides the first glimpses into the primary determinants of lysine recognition and how the riboswitch junction folds on ligand binding. Assuming that helix P1 is not fully formed before lysine binding and that protections in P5 are, at least in part, due to P1–P5 interactions, we propose that the main structural changes in solution, seen in both studies, involve stabilization of G80 and formation of the G12–C79 and G11–G163 junctional base pairs, followed by stabilization of the surrounding regions and the P1 helix (Supplementary Fig. 14). Prompted by pre-formation of tertiary riboswitch elements in solution, we have crystallized the riboswitch in the absence of lysine. This 3.1 Å structure (Supplementary Fig. 15) is very similar to the lysine-bound form, except that it lacks lysine and junctional K⁺. Although the absence of the expression platform and long P1 helix facilitate formation of this conformation, the structure emphasizes the importance of RNA interactions in maintaining the riboswitch conformation, suggests a crucial role of K⁺ in mediation of lysine-RNA but not RNA–RNA interactions, and reinforces the feasibility of lysine stabilizing a largely preformed riboswitch structure. The L box structure readily explains mutations that deregulate gene expression and confer resistance to AEC^{27,28} (Supplementary Fig. 16). The G12A, G12C and G81A mutations disrupt the lysine-binding pocket, whereas the G11A, G11U, G9C and C166U substitutions prevent pairing of the P1 helix. Therefore, intracellular lysine and AEC cannot bind the mutated riboswitches, and the segment downstream of G161 engages in formation of an anti-terminator stem (Supplementary Fig. 1), resulting in constitutive lysine production. The unusual architecture and high ligand specificity, achieved through a combination of shape complementarity and K⁺-assisted recognition of the bound lysine, distinguishes the lysine riboswitch from other riboswitches. Given the importance of lysine riboswitch-controlled gene expression for bacterial viability and the absence of the diaminopimelate pathway in mammals, the structure provides critical details towards facilitating the design of lysine-like analogues targeting riboswitches and other cellular sites.

METHODS

RNA preparation and complex formation

The lysine riboswitch, followed by the hammerhead ribozyme, was transcribed in vitro using T7 RNA polymerase. RNA was purified by denaturing polyacrylamide gel electrophoresis (PAGE) and anion-exchange chromatography. Lysine analogues were added to RNA at a 2.5–2.75 to 1 molar ratio. To form a complex without Mg²⁺, the RNA was mixed with 100 mM potassium-acetate, 1.0 mM EDTA and lysine. To prepare RNA for crystallization without lysine, 0.2 mM RNA was supplemented with 50 mM potassium acetate, pH 6.8, 50 mM sodium acetate, pH 6.9, and 2 mM MgCl₂, and concentrated twofold by Speedvac. Before crystallization, sodium-citrate, pH 5.7, was added to the mixture up to 100 mM, and the RNA sample was heated at 55 °C for 5 min and cooled on ice for 15 min.

Crystallization

Hanging drops were prepared by mixing 1 ml of the complex with 1 ml of the reservoir solution. The drops were equilibrated against 1 ml of reservoir solution at 20 °C for ~1–2 weeks. The riboswitch in the free state was crystallized in the solution containing 21% (w/v) PEG4000, 100 mM Bis-Tris, pH 5.5, and 25% isopropanol (v/v). For cryoprotection, crystals were quickly passed through the stabilizing solution, which was the reservoir solution with PEG4000 replaced by 20% PEG400. For soaking, crystals were passed through several 5 μ l drops of the stabilizing solution, and then incubated in 5 μ l of stabilizing solution supplemented with 3 mM [Ir(NH₃)₆]Cl₃, 10 mM CsCl, 10 mM thallium acetate, or 50 mM MnCl₂ salts for 7–8 h.

X-ray crystallography

Data were reduced using HKL2000 (HKL Research). The structure was determined using the autoSHARP option of SHARP and 2.4 Å MAD iridium data. The resulting experimental map was of excellent quality (Supplementary Fig. 17a) for most of the RNA molecule. The structure contains 1 RNA and 16 iridium hexamine sites (2 of them are split) per asymmetric unit (Supplementary Fig. 17b). The RNA model was built using 2.4 Å MAD electron density map, and then refined with REFMAC³¹ using 1.9 Å native data. The bound lysine and several cations with octahedral coordination geometry were added on the basis of the $2F_o - F_c$ and $F_o - F_c$ electron density maps. Cations were interpreted as Na⁺ or Mg²⁺ on the basis of the coordination distances in the range of 2.25–2.85 Å (Supplementary Fig. 17d) and 2.0–2.3 Å, respectively. Water molecules were added using ARP/wARP³². K⁺ cations were added on the basis of the 1.9 Å $2F_o - F_c$ and 2.9 Å anomalous (Supplementary Fig. 6) electron density maps and typical K⁺ coordination distances (Supplementary Fig. 17c). The final 1.9 Å riboswitch model contains 174 nucleotides, 1 lysine molecule, 1 magnesium cation, 3 potassium and 29 sodium cations. Cs⁺, Tl⁺, and Mn²⁺ cations were modelled on the basis of the anomalous maps (Supplementary Figs 7–9), whereas addition of other cations was guided by the high-resolution structure and the analysis of coordination geometries and distances.

Primer extension assay

Primer extension experiments were performed using 265-nucleotide full-length riboswitch. The ³²P-labelled 13-mer DNA oligonucleotide (100,000 c.p.m.), complementary to nucleotides 253–265 of RNA, was annealed to the 39 end of RNA (final RNA concentration 1 mM in the assay). Primer extension was conducted in 15 ml volume with 40 U of moloney murine leukaemia virus reverse transcriptase in 50 mM Na-HEPES, pH 7.9, 2 mM MgCl₂, and variable concentrations of NaCl and KCl (Fig. 4), with or without a tenfold excess of lysine over RNA. After 30 min incubation at 37 °C, the reactions were precipitated with ethanol, dissolved in loading buffer and analysed by 10% PAGE. Efficiency of lysine-induced pausing of reverse transcriptase at nucleotide A169 was quantified using FLA-7000 PhosphorImager and Image Gauge software (Fujifilm). Band intensities from independent experiments were averaged after gel-loading correction, background subtraction and normalization. The primer extension assay in the presence of lysine analogues was performed in 50 mM Na-HEPES, pH 7.9, 2 mM MgCl₂, 50 mM KCl, and lysine analogues in the range 10⁻⁸–10⁻² M. The data were fitted using a bimolecular equilibrium equation (Supplementary Fig. 12) and the resulting P50 values are reported in Fig. 4c. Reverse transcriptase sequencing reactions were run in parallel.

Footprinting studies

For footprinting experiments, the 174-nucleotide metabolite-sensing domain of the riboswitch was radioactively labelled at the 5' end by the kinase reaction. For inline

probing, 30,000–300,000 c.p.m. of 174-nucleotide RNA (1.6–16 nM) was incubated in 10–30 μ l solution containing 50 mM Tris-HCl, pH 8.3, 100 mM KCl and 20 mM MgCl₂ in the absence or presence of ~6–600-fold excess of lysine (Fig. 4) at room temperature for ~40 h. After incubation, aliquots were analysed by PAGE along with alkaline ladder and T1 nuclease digestion. For nuclease footprinting experiments, 20 μ l samples of 174-nucleotide RNA (100,000 c.p.m.) with a final RNA concentration 1 mM were preheated at 37 °C for 10 min in 50 mM Na-HEPES, pH 7.9, 50 mM KCl and 2 mM MgCl₂. Mixtures were incubated with a tenfold excess of lysine over RNA at 37 °C for 15 min. Cleavage reactions were performed with 0.0025 U RNase V1 (Pierce) or 0.2 U RNase T2 (Sigma) at 37 °C for 10 min. Reactions were quenched by the addition of 80 ml cold buffer and were immediately extracted with phenolchloroform and precipitated with ethanol. Radiolabelled RNA products were dissolved and analysed by PAGE.

Equilibrium dialysis

The assay was performed as described in ref. 1 using 5 kDa DispoEquilibrium DIALYZERS (Harvard Apparatus). In brief, 30 μ l RNA (from 0.001 to 20 mM) in the in-line probing buffer was placed in chamber A of the dialyser and equilibrated for 16 h at room temperature against chamber B containing 30 μ l ³H-labelled lysine (1 nM; ~6,000 c.p.m.) in the same buffer. The amount of bound lysine was calculated by subtracting the radioactivity counts of chamber B from chamber A. The data were fitted using a bimolecular equilibrium equation, assuming that the free lysine concentration is negligible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Overall structure and long-range tertiary interactions of the lysine-bound *T. maritima* riboswitch

a, Schematic of the riboswitch fold observed in the crystal structure of the complex. The bound lysine is in red. The RNA domains are depicted in colours used for subsequent figures. Base-specific tertiary contacts and long-range stacking interactions are shown as thin green and thick blue dashed lines, respectively. Nucleotides invariant in known lysine riboswitches are boxed. **b**, **c**, Overall lysine riboswitch structure in a ribbon representation showing front (**b**) and rotated by $\sim 60^\circ$ (**c**) views. **d**, The L2–L3 kissing loop interaction is formed by six base pairs, supplemented by interstrand stacking interactions between A42 and C95, G43 and U94, and G44 and G101. Hydrogen bonds between interstrand base pairs and orthogonally aligned G43 and U94 bases are depicted by dashed lines. **e**, The L4-loop–P2-helix interaction formed by an insertion of the A126–A127–A129 stack of L4 into the RNA groove of P2 distorted by noncanonical base pairs.

Figure 2. Structure and interactions in the junctional region of the lysine riboswitch
a, Stereo view of the junction with bound lysine. Green sphere depicts a K⁺ cation. **b**, Details of riboswitch lysine interactions. Lysine is positioned within the omit F_o-F_c electron density map contoured at 3.5 σ level. Water molecules are shown as light blue spheres. K⁺ cation coordination and hydrogen bonds are depicted by dashed lines. **c**, Direct and water-mediated interactions involving ϵ -ammonium group of lysine. **d**, **e**, Interactions in the top (**d**) and middle (**e**) junctional layers.

Figure 3. Interactions of lysine analogues with the riboswitch

a, Chemical structures of lysine and its analogues. **b**, Conformation and interactions of lysine analogues with the riboswitch. Lysine (red) and analogues (cyan) are superposed. Interactions between riboswitch and lysine analogues should be compared with lysine recognition in Fig. 2b. **c**, Cross-section through the surface view of the lysine-binding pocket showing the opening next to the carboxyl group of lysine (red arrow) and the free space next to the C4 atom (blue arrow). **d**, Cross-section through the homoarginine-riboswitch complex showing the opening (red arrow) next to the guanidinium group.

Figure 4. Probing lysine riboswitch tertiary structure

a, Primer extension analysis at various K⁺ concentrations. ³²P-labelled oligonucleotide was annealed to the 3' end of 265-nucleotide *T. maritima* RNA (1 mM) and extended by reverse transcriptase in the presence of 2 mM MgCl₂, at indicated concentrations of monovalent cations (in mM), and with or without a tenfold excess of lysine over RNA. **b**, Lysine binding affinity measured by equilibrium dialysis for riboswitches from *T. maritima* (top) and *B. subtilis* (bottom). Mg²⁺, K⁺ and Na⁺ concentrations are 20, 100 and 100 mM, respectively. The dissociation constants (mean ± s.d., mM; n=2–4) are: *T. maritima*, 0.10 ± 0.03 (Mg²⁺+K⁺), 4.14 ± 0.67 (Mg²⁺+Na⁺), 15.93 ± 0.09 (Mg²⁺); *B. subtilis*, 2.95 ± 0.30 (Mg²⁺+K⁺). **c**, The apparent ligand concentration at which reverse transcriptase pausing is half-maximally attained (P_{50}) (n=2) in the primer extension experiments. HArg, L-homoarginine; IEL, iminoethyl-L-lysine; Lys, L-lysine; Oxa, L-4- oxalysine. **d**, In-line probing of 5' ³²P-labelled *T. maritima* 174-nucleotide RNA (1.6 nM) in the absence and presence of lysine (1.1 mM). T1 and 2OH designate RNase T1 and alkaline ladders, respectively. NR, no reaction. Strong and weak cleavage reductions are shown in red and pink colours, respectively. **e**, **f**, Probing of 174-nucleotide RNA (1 mM) by V1 and T2 nucleases with or without a tenfold excess of lysine. Weak and strong cleavage enhancements are shown in light and dark green, respectively, in **e**. **g**, Strong lysine-induced

cleavage reductions are colour coded in the riboswitch structure. Light orange, green and red are reductions identified in ref. 1 using *B. subtilis* RNA with a short P1 helix, overlapping reductions of the present study and in ref. 1, and extra reductions found in the present study, respectively.