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The Dynamic Nature of RNA as Key to Understanding Riboswitch Mechanisms

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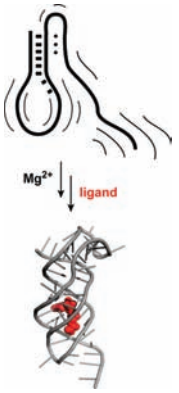
CONSPECTUS

Riboswitches are gene regulation elements within RNA that recognize specific metabolites. They predominantly occur in the untranslated leader regions of bacterial messenger RNA (mRNA). Upon metabolite binding to the aptamer domain, a structural change in the adjoining downstream expression platform signals "on" or "off" for gene expression.



Researchers have achieved much progress in characterizing ligand-bound riboswitch states at the molecular level; an impressive number of high-resolution structures of aptamer–ligand complexes is now available. These structures have significantly contributed toward our understanding of how riboswitches interact with their natural ligands and with structurally related analogues. In contrast, relatively little is known about the nature of the unbound (apo) form of riboswitches. Moreover, the details of how changes in the aptamer domain are transduced into conformational changes in the decision-making expression platform remain murky.

In this Account, we report on recent efforts aimed at the characterization of free states, ligand recognition, and ligand-induced folding in riboswitches. Riboswitch action is best approached as a cotranscriptional process, which implies sequential folding and release of the aptamer prior to the signaling of the expression platform. Thus, a complex interplay of several factors has to be taken into account, such as speed of transcription, transcriptional pausing, kinetics and thermodynamics of RNA structure formation, and kinetics and thermodynamics of ligand binding. The response mechanism appears to be best described as a process in which ligand recognition critically dictates the folding pathway of the nascent mRNA during its expression; the resulting structures determine the interactions with the transcriptional or translational apparatus.

We discuss experimental methods that offer insight into the dynamics of the free riboswitch state. These include probing experiments, such as in-line and selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) techniques, small-angle X-ray scattering (SAXS) analysis, NMR spectroscopy, and fluorescence spectroscopy, including single-molecule fluorescence resonance energy transfer (smFRET) imaging. One of our research contributions is an approach, termed 2ApFold, that incorporates noninvasive 2-aminopurine modifications in riboswitches. The fluorescence response of these moieties is used to delineate the order of secondary–tertiary structure formation and rearrangements taking place during ligand-induced folding. This information can be used to explore the kinetics of ligand recognition and to analyze the degree of structure preorganization of the free riboswitch state.

Furthermore, we discuss a recent smFRET study on the SAM-II riboswitch; this report underscores the importance of choosing strategic labeling patterns that leave maximal conformational freedom to the regulatory interaction. Finally, we comment on how riboswitch ligand recognition appeals to the concepts of conformational selection and induced fit, and on the question of whether riboswitches act under thermodynamic or kinetic control.

This Account highlights the fact that a thorough understanding of RNA dynamics *in vitro* is required to shed light on cellular riboswitch mechanisms. Elucidating these mechanisms will contribute not only to ongoing efforts to target riboswitches with antibiotics but also to attempts to engineer artificial cell regulation systems.

What Is the Relationship between Riboswitch Structure Formation and Ligand Binding?

Riboswitches function as gene regulatory elements that can modulate transcription, translation, splicing, or RNA stability.[1,2] They are located in noncoding regions of mRNA and recognize metabolites with high selectivity. As a consequence, mutually exclusive structures of the riboswitch are adopted, corresponding to the metabolite-bound and

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unbound RNA that signal "on" or "off" to the machinery of gene expression.[3−6] The majority of riboswitches act in *cis* with only one known exception for a S-adenosylmethionine (SAM) sensitive system in *Listeria monocytogenes*.[7]

This simplified picture, however, could not explain why the "full-length" domains (comprising both the ligand-sensitive aptamer and the adjoining expression platform) of several riboswitch classes were incapable of ligand recognition in *in vitro* binding assays while their aptamer domains alone bound to their ligands with high affinity.[8−11] Additionally, for many distinct riboswitches, the available cellular metabolite concentrations are significantly higher than actually required for complete binding, as highlighted by lower than expected K_d-values for metabolite complexes.[11,12] To comprehend these findings which, at first sight, seem contradictory to the concept of riboswitches, their action has to be elucidated in the light of being a cotranscriptional process.[13−16] Riboswitch transcription implies sequential folding, releasing the aptamer prior to the expression platform, and thus a complex interplay of several factors such as speed of transcription, transcriptional pausing, and kinetics and thermodynamics of RNA structure formation and of ligand binding have to be taken into account.[10,14] The response mechanism seems to be better described as a process where ligand recognition critically dictates the folding pathway of the nascent mRNA during its expression and the resulting structures determine the interactions with the transcriptional or translational apparatus. From these considerations, one of the most intriguing questions in the riboswitch field asks for the relationship between riboswitch structure formation and ligand binding, and how ligand binding is transposed into a regulatory signal.

Which Aspects of RNA Dynamics Are Crucial for Riboswitch Ligand Recognition?

A prerequisite for riboswitch function is that the ligand-free and ligand-bound riboswitch states do not find themselves in dynamic equilibrium with each other, meaning that they do not exchange rapidly on a time scale relevant to gene control. Furthermore, the cell must have evolved pathways that allow for sufficient flexibility of the nascent RNA chain to retain competence for ligand recognition and on the other hand guarantee the ability to enter a default folding pathway in the absence of ligand which results in a different secondary structure outcome. Hence, the ligand-recognizing competent structure, although getting close to the ligand-bound conformation, is not allowed to turn into a thermodynamically stable trap that blocks the alternative folding pathway and regulatory outcome. In other words, becoming a trap must solely apply to the final complex which is achieved by capturing the ligand-recognizing competent structures of the nascent riboswitch RNA through ligand binding.

The enormous progress in characterizing ligand-bound riboswitch states at the molecular level is reflected in the impressive number of high-resolution structures of riboswitch aptamer/ligand complexes that have been determined in the past 7 years and that have significantly contributed to understand the concepts of how riboswitches interact with cognate and near-cognate ligands.[17−27] In comparison, relatively little is known about the nature of the apo-form of riboswitches during the ligand-sensing phase and how changes in the aptamer domain are transduced into conformational changes in the decision-making expression platform. So far, previous studies on this topic have presented an inconsistent picture. Many ligand-bound sensing domains of riboswitches seclude the ligands inside their binding pockets. Access of the ligand to the pocket must therefore proceed via binding of the ligand to partially unfolded riboswitch molecules. On the other hand, crystal structures of riboswitches in the ligand-free state[19,20,27,28] clearly indicate that riboswitches are capable of folding into a near "ligand-bound" conformation without actual ligand binding. Therefore, the question on how much RNA should be unfolded or folded prior to the specific metabolite recognition event remains largely unanswered.

What Experimental Methods Grant Insights into the Free Riboswitch State?

Characterization of the free riboswitch state turned out to be much more difficult compared to their bound counterparts because of a high degree of intrinsic conformational heterogenity. Specific insights into the structural differences between the two forms can be rapidly obtained by those chemical probing techniques that allow investigation of all nucleotides at a time. Besides "in-line" probing, which relies on the inherent intramolecular 2′-OH reactivity for phosphodiester transesterification and the analysis of the induced strand cleavage patterns,[29] selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) has become a valuable tool.[30,31] The latter approach employs *N*-methylisatoic anhydride or benzoyl cyanide as probing reagent that selectively modifies 2′-hydroxyl groups within the regions where the backbone is conformationally flexible. Conversely, nucleotides that are locked in helices or

highly structured tertiary interactions display low reactivity. Additionally, the value of the method is further expressed by its temperature-dependent version allowing the identification of apparent melting temperatures (T_m) for most nucleotide positions in the RNA. For the SAM-I riboswitch, the difference between T_m values in the presence and absence of SAM revealed the largest stability changes in the tertiary architecture around a central base triple that anchors two junctions and a pseudoknot followed by the region of the SAM binding pocket.[28,32] In another example, SHAPE analysis of the free and unbound thiamine pyrophosphate sensing (TPP) riboswitch aptamer emphazised that, in the ligand-free state, the aptamer consists of five well-formed and stable helices that are linked by highly dynamic single-stranded regions, suggestive of a structure with little or no stable tertiary interactions.[33] Noteworthily, hydroxyl radical footprinting and UV-cross-linking have also been alternatively applied to verify preorganized states of the glmS riboswitch in the absence of ligand.[34]

While biochemical probing techniques can provide a wealth of information on nucleotides and sequence stretches of an unliganded riboswitch that interact with each other, they yield limited information about the overall architecture and, in particular, the interhelical organization. To provide more complete models, small-angle X-ray scattering (SAXS) analysis has been employed for several riboswitch aptamers.[25,28,35−38] The SAXS approach takes advantage of the fact that the scattering of X-rays is sensitive to the overall shape of a macromolecule in solution. If the free state RNA samples multiple conformations in solution, computational approaches can be used to identify a unique set of conformational states that best describes the observed X-ray scattering profile. In the case of the SAM-I aptamer, such an ensemble optimization was generated in the presence of only Mg^{2+} and yielded a solution state defined by multiple alternative conformations.[28] Nevertheless, a subdomain consisting of two helices (P4 and P2a) and the pseudoknot was nearly identical in all of them and comparable to the arrangement in the crystal structure of the bound state. Other helices (P1 and P3) were significantly twisted relative to the subdomain. Moreover, for a small subgroup of the modeled structures of the free-state ensemble, all helical segments were positioned as they are in the crystal structure, indicating that this conformation is accessible in the absence of ligand. In the case of the cyclic diguanylate and the TPP riboswitch aptamers, SAXS revealed more distinct states of free and bound conformations.[25,35] Mg^{2+} alone, without the ligand, was insufficient to stabilize the globally compact form, and the extended shape of the free state was significantly divergent compared to the shape observed for the bound state.

Although SAXS can be helpful in suggesting the global arrangement of structural segments, this approach lacks high resolution. X-ray crystallography would warrant high resolution, but crystal structures of free riboswitch domains, in most cases, reflect a minor population of the actual ensemble in solution, and this method therefore delivers an incomplete picture. Consequently, great efforts have been put in employing nuclear magnetic resonance (NMR) methods to receive atomic resolution information for free states. Despite the challenging sizes of riboswitch RNA for this technique, important insights have been obtained, in particular in the case of purine riboswitches.[39−43] In the presence of physiological concentrations of Mg^{2+}, the three-way junction aptamer takes the same global shape with and without the ligand. This includes the parallel orientation of stems P2/P3 and the long-range loop−loop L2/L3 interaction. In contrast, the ligand-binding core region of the free state of the purine riboswitch appears largely disordered and remains the crucial factor to allow for recognition of the ligand. It is also noteworthy that a single-molecule optical tweezer study on the adenine riboswitch is in accordance with hierarchical folding and supports the model obtained by NMR spectroscopy.[44]

NMR spectroscopy was further successfully applied to achieve direct evidence for the secondary structure of the SAM-III riboswitch in the free state which significantly differed from the one in the ligand-bound state.[45] These examples demonstrate that NMR can be very useful to ascertain preorganized structures of an unbound riboswitch with high accuracy.

What Is the Relevant Time Scale for the Riboswitch−Ligand Interaction?

Investigations on kinetics of ligand binding and accompanying RNA folding open another important dimension in riboswitch studies and have, so far, mostly involved fluorescence spectroscopy. Early studies exploited the fact that flavin mononucleotide (FMN) is fluorescent and can be sensed by a riboswitch.[11] Likewise, 2-aminopurine, a structurally close analogue of adenine, is fluorescent and has a nearly equal binding affinity to the adenine riboswitch.[8] Fluorescence spectroscopy studies on these systems revealed the first precise on-rates, in the order of 10^4−10^5 M^{-1} s^{-1}, and independently determined off-rates, in the order of 10^{-2}−10^{-3} s^{-1}.[8,11] More recent studies confirmed

these ranges for the majority of riboswitch classes,[14,46] with the exceptions of preQ$_1$-class I,[47,48] which presented a faster response ($k_{on} \sim 6 \times 10^3$ M^{-1} s^{-1}), and cyclic diguanylate riboswitch,[24] with a significantly slower off-rate ($k_{off} \sim 2 \times 10^{-7}$ s^{-1}). (For a complete kinetics parameters table, see the Supporting Information of ref 47.)

Additionally, in a recent NMR study using advanced ultraSOFAST pulse sequences,[43] the conformational transitions of an adenine riboswitch aptamer were revealed in real-time (with 1 s time shots) and with atomic resolution. It was observed that, following the initial recognition steps of the ligand by the core of the three-way junction, long-range loop−loop interactions are created and then progressively stabilized before the formation of a fully stable complex over a time course of approximately 2−3 min. With respect to reported values for the speed of transcription in cells (10−200 nt/s),[49,50] the actual recognition process can be considered slow and transcriptional pausing may be required to "wait" for aptamer folding and ligand binding to avoid misfolded, nonresponsive structures.

What Insights Are Provided by 2-Aminopurine Based RNA Folding Analysis (2ApFold)?

In our research program, we have developed a fluorescent spectroscopic approach (termed here 2ApFold) that assists in the assessment of folding kinetics during ligand-induced RNA structure rearrangements (Figure 1).[10,47,51−53] A major aim was to obtain precise kinetic data of the formation of secondary and tertiary interactions in individual structural segments to disclose a likely order of structure formation events (Figure 2A). This approach uses the power of chemical synthesis to site-specifically label RNA with the minimally invasive fluorescent nucleobase 2-aminopurine (2Ap).[54,55] In combination with enzymatic ligation, the typical sizes (50−100 nt) of most riboswitch aptamers are efficiently accessible.[52,53,56] To select suitable positions for nucleoside replacements with 2Ap, the three-dimensional structure of the riboswitch is analyzed following the criteria of retaining hydrogen-bonding patterns and of maintaining highly conserved sequence portions. By doing this, about 10−15% of nucleosides within the sequence can be identified that participate in crucial secondary and tertiary structure interactions and that fulfill the above-mentioned criteria (Figures 1B,C; 2A). If the crystal structure is not available, we found that SHAPE analysis provides a helpful tool since those nucleosides that become more flexible in the metabolite-bound RNA usually correspond with nucleosides that are looped out or at least partially unstacked.[18,32]

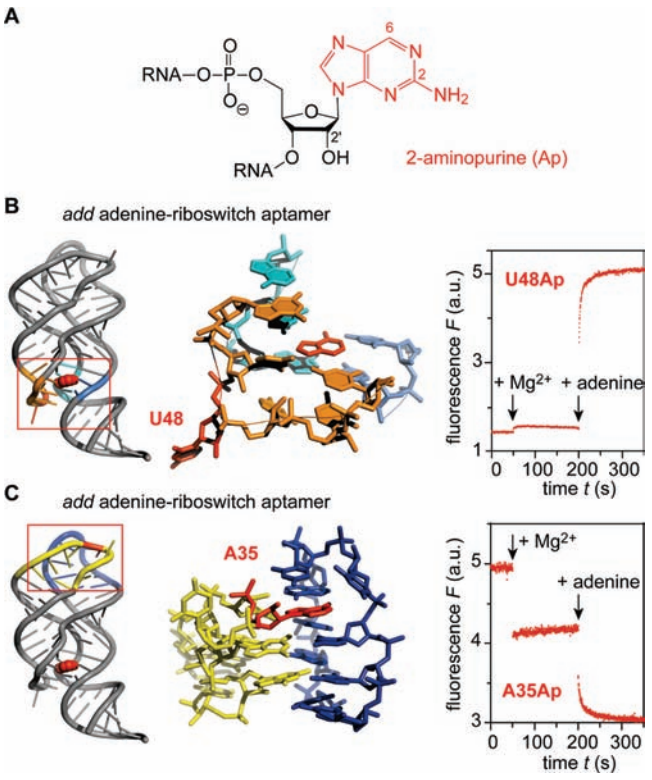

FIGURE 1. 2-Aminopurine (2Ap) labeling to study Mg^{2+}- and ligand-induced RNA folding (2ApFold approach). (A) Chemical structure of 2Ap. (B,C) Structure-based selection of 2Ap nucleoside replacement exemplified for U48 (B) and A35 (C) of the adenine riboswitch, and corresponding fluorescence response.

Upon replacement of the selected nucleosides by 2Ap, a fluorescence variation in a structurally important region for which this label becomes the representative "rate sensor" is observed.[46,51] So far, using this approach, riboswitches that sense adenine and guanine,[10] TPP,[46] preQ$_1$,[47] and SAM[51] have been investigated. We demonstrated that an order of structure folding was observable, with rate constants between the individual regions varying about 3-fold for the adenine aptamer,[10] 2-fold for the SAM-II aptamer,[51] and almost 7-fold for the TPP aptamer (Figure 2A).[46]

In addition, the 2ApFold approach allows determination of binding constants for the metabolite/riboswitch complexes. The values obtained usually compare well with the ones determined by other methods such as isothermal microcalorimetry (ITC).[10,46,47,51]

Furthermore, the 2ApFold approach is of high value for investigations of riboswitch full-length domains,[10,46,47,51−53] including both the aptamer and the expression platform (Figure 2B). For example, comparison of 2ApFold data for the transcription-controlling *pbuE* A-riboswitch and the translation-controlling *add* A-riboswitch revealed that the latter remains fully responsive to ligand binding (Figure 2B,

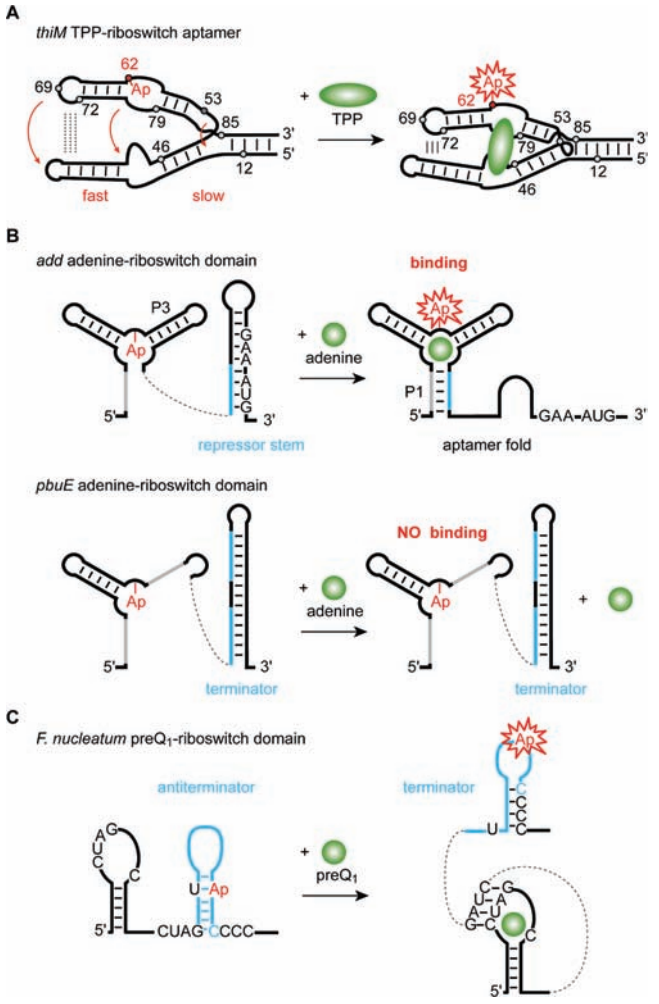

FIGURE 2. Selected applications of the 2ApFold approach to study riboswitch-ligand recognition. (A) 2Ap-labeled TPP riboswitch aptamer: following the time course of fluorescence changes reveals the order of ligand-induced tertiary structure formation.[46] (B) 2Ap-labeled adenine riboswitch domains to reveal distinct ligand-dependent fluorescence reponses.[10] (C) 2Ap-labeled $preQ_1$-class I riboswitch domain to determine kinetics of ligand-induced rearrangement from antiterminator to terminator structures.[47]

upper panel) while the former does not (Figure 2B, lower panel).[10] The *add* A-riboswitch's sequence therefore carries the intrinsic property to act under thermodynamic control *in vivo* (Figure 3A). In contrast, the full-length *pbuE* A-riboswitch which uses the same aptamer architecture becomes trapped in a nonresponsive terminator fold, resisting ligand binding *in vitro* (Figure 2B, lower panel). The *pbuE* A-riboswitch must therefore be kinetically controlled *in vivo*,[10] likely involving transcriptional pausing that ascertains enough time for the aptamer to fold into a ligand-binding competent structure that can be captured by the ligand prior to formation of the extraordinarily stable terminator stem-loop (for *pbuE* transcription OFF, see Movie 1 and Figure 3B, upper panel; for transcription ON, see Movie 2 and Figure 3B; lower panel) and/or by using high metabolite concentrations to increase ligand binding rates.

More recently, we demonstrated another application of the 2ApFold approach, namely, the direct kinetic assessment of the antiterminator-terminator rearrangement in a transcriptionally controlled $preQ_1$ sensing riboswitch (Figure 2C).[47,48] The full-length domain of this riboswitch was shown to exist in a bistable equilibrium of competing antiterminator and terminator hairpins. The sizes of the competing stems comprise four versus six base pairs and imply an intrinsic exchange rate of minimal ~0.005 to maximal ~0.1 s^{-1} according to recent studies.[57−60] At the transcriptional time scale, such hairpins thus do not exchange and rather exist in an "either antiterminator or terminator" fashion. Consistent with this, the ligand-induced shift from one structure to the other was found to proceed significantly faster than the expected inherent exchange rate. Using the 2ApFold approach with an appropriately positioned fluorescent label in the bistable segment of the full-length riboswitch (Figure 2C), the rate for this process was in the order of 2 s^{-1}.[47]

The final aspect of 2ApFold to be discussed here concerns Mg^{2+}-induced preorganization of free riboswitches. For the SAM-II riboswitch,[61] we demonstrated that the individually 2Ap labeled variants responded to increasing concentration of Mg^{2+} with clean sigmoid transitions on a logarithmic scale at physiologically relevant concentrations (1−5 mM).[51] Strikingly, the distinct midpoints (reminiscent of T_m values of UV-melting profiles) are suggestive to correspond to the different degree of preorganization in the structure. This correlation, however, awaits further investigations involving other riboswitch classes to validate the hypothesis.

Why is SAM-II RNA Inspiring for smFRET Studies of Other Riboswitches?

So far, the majority of efforts to understand the ligand-induced folding of riboswitches have focused on RNAs, such as purine[8−10,39−43] and SAM-I riboswitches,[28,32] that form three- or four-way junctions closed by the "regulatory" helix which comprises the crucial interface between aptamer and expression platform. These studies, in many instances, cannot adequately answer the still outstanding questions, since the RNA constructs used in the experiments typically contained a metabolite-sensing domain closed by a stabilized regulatory helix that preformed prior to the ligand binding. Therefore, these experiments are set to understand

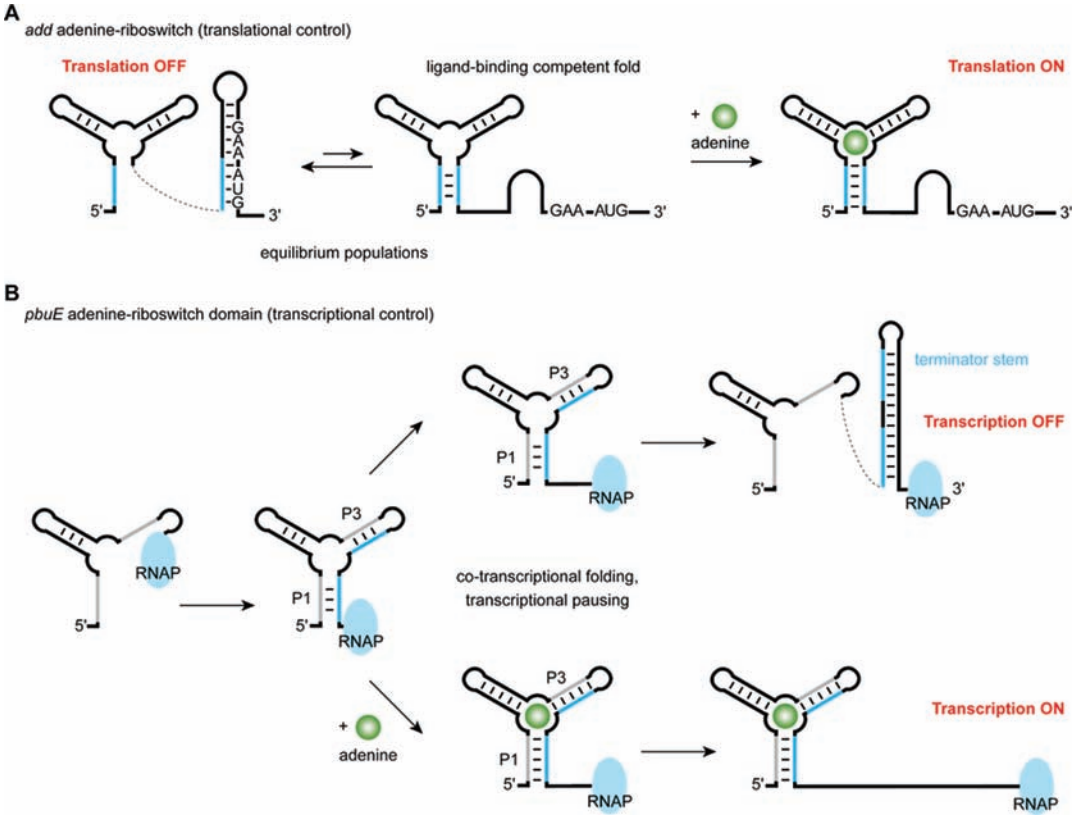

FIGURE 3. Possible *in vivo* scenarios for ligand-induced response mechanisms of adenine riboswitches at the translational (A) and transcriptional (B) levels, based on observations from the 2ApFold approach (see Figure 2B).[10] For discussion, see main text.

conformational sampling limited by the framework of stable RNA constructs.

The SAM-II and the SAM-III riboswitches are exceptional in the sense that they control gene expression at the level of translation initiation using the ribosome-entry site embedded in the metabolite-sensing domain.[18,45,61] This means that the minimal aptamer motif includes the expression platform at the very 3′-end. In a recent comprehensive study, we explored the ligand-induced folding process of the SAM-II riboswitch using NMR and fluorescence spectroscopy, and single-molecule fluorescence resonance energy transfer (smFRET) imaging.[51] The data revealed that the unliganded SAM-II riboswitch is highly dynamic in nature, where its conserved stem-loop element becomes engaged in a pseudoknot fold through base-pairing with nucleosides in the single-stranded 3′-overhang. The pseudoknot structure is highly transient in the absence of its ligand, *S*-adenosylmethionine (SAM), and becomes conformationally restrained upon ligand recognition (for SAM-II translation ON, see Movie 3; for translation OFF, see Movie 4). The smFRET experiments revealed the underlying dynamics of pseudoknot sampling in the free state to be in the hundreds of milliseconds. To achieve a highly reliable smFRET data set, the modification pattern was chosen in a way to immobilize the riboswitch at the least dynamic segment, namely, the hairpin stem which is preorganized even in the absence of Mg^{2+}. Importantly, this immobilization did not compromise the crucial pseudoknot region of the molecule that was expected to be dynamic. Attaching fluorophores at the Shine–Dalgarno sequence and the hairpin loop were therefore ideally positioned to directly sense the regulatory interaction, namely pseudoknot formation (Figure 4A).

So far, smFRET investigations on riboswitches have been reported only in the context of the purine sensing aptamer domain.[9,62] In these constructs, however, the regulatory helix was used as anchor for immobilization and thereby stabilized by increasing the number of base pairs. The labeling patterns used in these examples were appropriate to confirm that the loop–loop interaction is preorganized and therefore less dynamic (Figure 4B). However, they were inappropriate to reveal potential dynamics of the crucial regulatory helix. The chemical handles used for the SAM-II riboswitch suggest that an analogous labeling pattern for the purine systems should be promising to unveil dynamics of their regulatory helices.

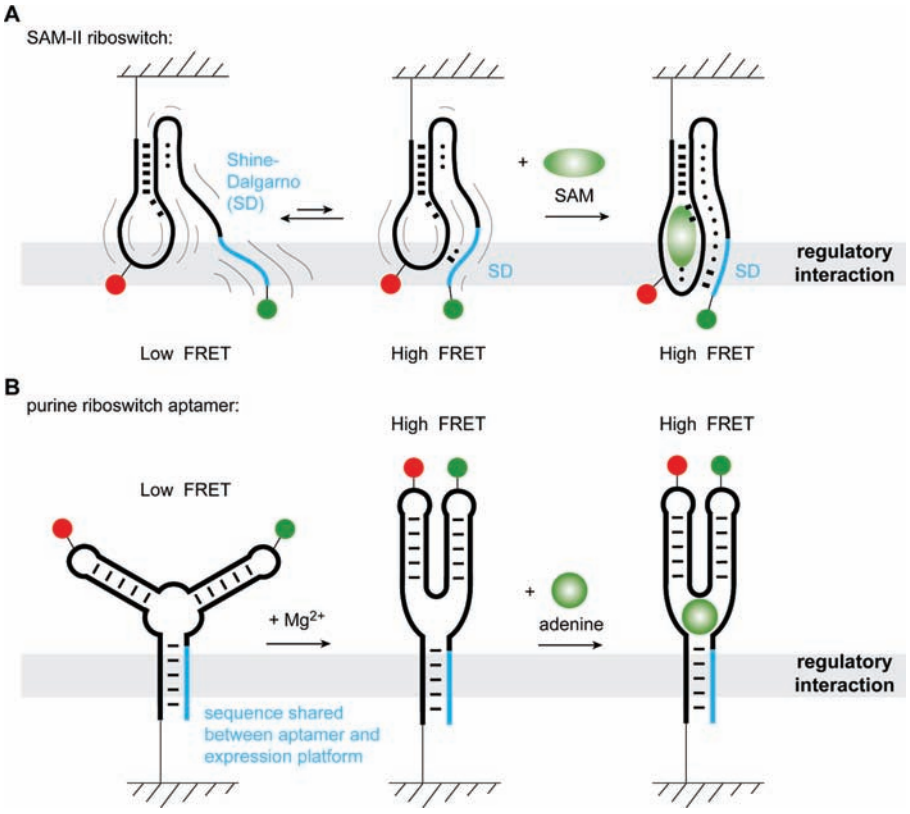

FIGURE 4. Single-molecule FRET studies. (A) SAM-II riboswitch.[51] The chemical handles were attached to directly sense the regulatory event, namely, pseudoknot formation. (B) Purine riboswitch.[9,62] Labeling pattern to sense the Mg^{2+}-induced RNA preorganization.

How Does Riboswitch–Ligand Recognition Appeal to the Concepts of "Induced Fit" and "Conformational Selection"?

The hypothesis of "induced fit" recognition claims that conformational states between the free and bound configurations are driven toward the bound-state structure by the nature of the binding interaction.[63] Contrarily, the "lock and key" hypothesis states that binding is driven by inherent structural complementarities between the ligand and the target. In recent years, the concept of "conformational selection" has emerged based on the evidence of the intrinsically dynamic nature of biomolecules.[64–67] This model postulates that all conformations of a biomolecule are pre-existing in a dynamic ensemble including binding competent conformations, being present at least in minor populations. The ligand selects these populations, and consequently, a redistribution of the relative conformational sub-states occurs (Figure 5).

With respect to riboswitches, these concepts are currently intensively discussed. A recent study of the free state of the four-way junction forming SAM-I riboswitch aptamer suggested that Mg^{2+} initiates the collapse from an RNA with only secondary structure to a conformationally restricted ensemble

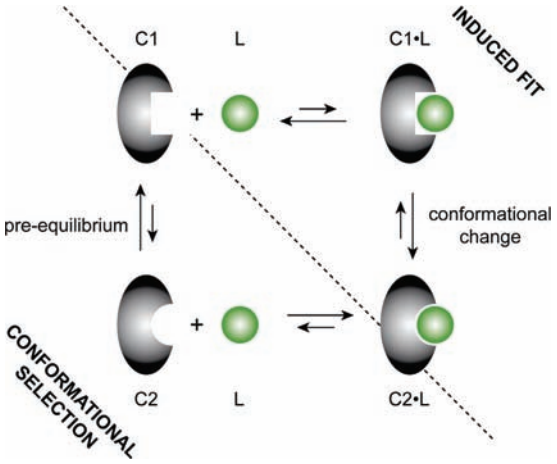

FIGURE 5. Two models for molecular recognition: induced fit and conformational selection.[63] In conformational selection, the binding-competent conformation (C2) is pre-existing in solution before the addition of ligand (L). In induced fit, initial binding contacts (C1•L) between ligand and receptor induce conformational rearrangements to achieve the conformation C2•L of the complex.

with distinct tertiary interactions.[28] It was proposed that the aptamer rapidly samples conformations on both the global and the local scale during the sensing phase.[28,32] In the structurally distinct SAM-III riboswitch, the structures of ligand-free and

ligand-bound states were shown to differ significantly.[45] Based on the evidence of a minor population of the ligand-free RNA with the same secondary structure as the SAM-bound form,[45] a conformational selection mechanism was postulated.

In the case of purine riboswitches, NMR[39−43] and single-molecule FRET studies[9,62] have shown that the three-way junction adopts the same global shape at physiological Mg^{2+} concentrations with and without the ligand. However, the ligand-binding core region was shown to remain largely disordered in the absence of ligand. This feature of the purine riboswitch was postulated to be a critical determinant of ligand recognition, and described as an interplay of induced fit and preorganization[39] or a predetermined-induced fit mechanism.[41]

The free SAM-II riboswitch forms a minimal hairpin structure in the absence of ligand and Mg^{2+} cations and is capable of adopting near ligand-bound conformation upon addition of magnesium.[51] Single-molecule FRET experiments clearly documented the fluctuation of this riboswitch between the two ensemble states of a hairpin and a "loose pseudoknot", with the latter being structurally close to the final SAM-bound RNA complex. The "loose pseudoknot" stands for the ligand-binding competent, selected conformation (Figure 4). In addition, fluorescence time course experiments of the individual 2Ap-modified variants in response to SAM further suggest that once the initial recognition events between SAM and the selected conformer have occurred, local conformational adjustments take place that are even sensed in the nucleotide regions that are distant from the actual site of binding.[51] Such features are consistent with an "induced fit" but can also be appropriately described by an adaptive recognition process via a "conformational selection-type" mechanism.[68,69]

Signal Transduction in Riboswitches: Thermodynamic or Kinetic Control?

Transduction of ligand binding into a genetic decision can occur under kinetic or thermodynamic control.[8,10,11] The relevant parameters have been excellently reviewed[14] and can concern the rate of aptamer folding, ligand concentration, k_{on}, k_{off}, and K_d of the ligand/aptamer complex, speed of transcription, delay time at transcriptional pauses, competition between alternative RNA structures, and the height of activation energy barrier between bistable structures. In short, while in a kinetically controlled scenario, the kinetic parameters of transcription are adequately geared to those of ligand binding and cotranscriptional folding of the aptamer, the thermodynamic scenario depends on the equilibrium between ligand association and dissociation, or between bistable, interconverting structures whose populations are shifted by the ligand interaction.

The definition of thermodynamic/kinetic control refers to the transcriptional time between complete synthesis of the aptamer by the RNA polymerase (RNAP) and its progression to the termination decision point (Δt_{RNAP}),[8,11,14,47] for example, by formation of the terminator stem-loop. If this time is comparable to or longer than $1/k_{off}$, the switch will approximate equilibrium control. However, if Δt_{RNAP} is much smaller than $1/k_{off}$, the switch is under kinetic control. This means that the RNAP arrives at the termination decision point before the ligand and the aptamer reach binding equilibrium. Because the equilibrium is not reached, a ligand concentration equivalent to K_d does not lead to 50% ligand binding, resulting in discrepancies between the half-maximal shift in termination efficiency (T_{50}) and K_d.[11,14]

The typical time constants τ ($\sim 1/k_{off}$) for riboswitch association and dissociation equilibria are in the range of seconds (time required for e^{-1} of the reaction to go toward completion).[14] This is comparable to the time of elongation of RNA polymerase from the end of the aptamer domain to the termination decision point and shows that both regimes are in principle accessible for cellular systems by tuning the length of the expression platform, elongation speed, and dwell times at pause sites.

Additionally, many other factors such as nutrititional availability that would alter intracellular NTP levels or possible RNA chaperone activity on the nascent RNA chain make riboswitch signal transduction a most complex process that warrants more research to comprehend the thus far well hidden coherences.

BIOGRAPHICAL INFORMATION

Andrea Haller was born in 1985 in Kufstein, Austria. She is currently working toward her Ph.D. degree in chemistry at the University Innsbruck. Her research interests include riboswitches and synthetic RNA chemistry.

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FOOTNOTES

†These authors contributed equally.

REFERENCES

1 Roth, A.; Breaker, R. R. The structural and functional diversity of metabolite-binding riboswitches. *Annu. Rev. Biochem.* **2009**, *78*, 305–334.

2 Lee, E. R.; Baker, J. L.; Weinberg, Z.; Sudarsan, N.; Breaker, R. R. An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science* **2010**, *329*, 845–848.

3 Montange, R. K.; Batey, R. T. Riboswitches: emerging themes in RNA structure and function. *Annu. Rev. Biophys.* **2008**, *37*, 117–133.

4 Blouin, S.; Mulhbacher, J.; Penedo, J. C.; Lafontaine, D. A. Riboswitches: Ancient and promising genetic regulators. *ChemBioChem* **2009**, *10*, 400–416.

5 Mironov, A. S.; Gusarov, I.; Rafikov, R.; Lopez, L. E.; Shatalin, K.; Kreneva, R. A.; Perumov, D. A.; Nudler, E. Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. *Cell* **2002**, *111*, 747–756.

6 Schwalbe, H.; Buck, J.; Fürtig, B.; Noeske, J.; Wöhnert, J. Structures of RNA switches: Insight into molecular recognition and tertiary structure. *Angew. Chem., Int. Ed.* **2007**, *46*, 1212–1219.

7 Loh, E.; Dussurget, O.; Gripenland, J.; Vaitkevicius, K.; Tiensuu, T.; Mandin, P.; Repoila, F.; Buchrieser., C.; Cossart, P.; Johansson, J. A trans-acting riboswitch controls expression of the virulence regulator PrfA in Listeria monocytogenes. *Cell* **2009**, *139*, 770–779.

8 Wickiser, J. K.; Cheah, M. T.; Breaker, R. R.; Crothers, D. M. The kinetics of ligand binding by an adenine-sensing riboswitch. *Biochemistry* **2005**, *44*, 13404–13414.

9 Lemay, J. F.; Penedo, J. C.; Tremblay, R.; Lilley, D. M.; Lafontaine, D. A. Folding of the adenine riboswitch. *Chem. Biol.* **2006**, *13*, 857–868.

10 Rieder, R.; Lang, K.; Graber, D.; Micura, R. Ligand-induced folding of the adenosine deaminase A-riboswitch and implications on riboswitch translational control. *ChemBioChem* **2007**, *8*, 896–902.

11 Wickiser, J. K.; Winkler, W. C.; Breaker, R. R.; Crothers, D. M. The speed of RNA transcription and metabolite binding kinetics operate an FMN riboswitch. *Mol. Cell* **2005**, *18*, 49–60.

12 Garst, A. D.; Batey, R. T. A switch in time: detailing the life of a riboswitch. *Biochim. Biophys. Acta* **2009**, *1789*, 584–591.

13 Xayaphoummine, A.; Viasnoff, V.; Harlepp, S.; Isambert, H. Encoding folding paths of RNA switches. *Nucleic Acids Res.* **2007**, *35*, 614–622.

14 Zhang, J.; Lau, M. W.; Ferré-D'Amaré, A. R. Ribozymes and riboswitches: modulation of RNA function by small molecules. *Biochemistry* **2010**, *49*, 9123–9131.

15 Garst, A. D.; Edwards, A. L.; Batey, R. T. Riboswitches: Structures and Mechanisms. *Cold Spring Harbor Perspect. Biol.* **2010**, DOI: 10.1101/cshperspect.a003533.

16 Al-Hashimi, H. M.; Walter, N. G. RNA dynamics: it is about time. *Curr. Opin. Struct. Biol.* **2008**, *18*, 321–329.

17 Serganov, A. Determination of riboswitch structures: light at the end of the tunnel? *RNA Biol.* **2010**, *7*, 98–103.

18 Gilbert, S. D.; Rambo, R. P.; Van Tyne, D.; Batey, R. T. Structure of the SAM-II riboswitch bound to S-adenosylmethionine. *Nat. Struct. Mol. Biol.* **2008**, *15*, 177–182.

19 Garst, A. D.; Héroux, A.; Rambo, R. P.; Batey, R. T. Crystal structure of the lysine riboswitch regulatory mRNA element. *J. Biol. Chem.* **2008**, *283*, 22347–22351.

20 Serganov, A.; Huang, L.; Patel, D. J. Structural insights into amino acid binding and gene control by a lysine riboswitch. *Nature* **2008**, *455*, 1263–1267.

21 Klein, D. J.; Edwards, T. E.; Ferré-D'Amaré, A. R. Cocrystal structure of a class I $preQ_1$ riboswitch reveals a pseudoknot recognizing an essential hypermodified nucleobase. *Nat. Struct. Mol. Biol.* **2009**, *16*, 343–344.

22 Kang, M.; Peterson, R.; Feigon, J. Structural Insights into riboswitch control of the biosynthesis of queuosine, a modified nucleotide found in the anticodon of tRNA. *Mol. Cell* **2009**, *33*, 784–790.

23 Serganov, A.; Huang, L.; Patel, D. J. Coenzyme recognition and gene regulation by a flavin mononucleotide riboswitch. *Nature* **2009**, *458*, 233–237.

24 Smith, K. D.; Lipchock, S. V.; Ames, T. D.; Wang, J.; Breaker, R. R.; Strobel, S. A. Structural basis of ligand binding by a c-di-GMP riboswitch. *Nat. Struct. Mol. Biol.* **2009**, *16*, 1218–1223.

25 Kulshina, N.; Baird, N. J.; Ferré-D'Amaré, A. R. Recognition of the bacterial second messenger cyclic diguanylate by its cognate riboswitch. *Nat. Struct. Mol. Biol.* **2009**, *16*, 1212–1217.

26 Edwards, A. L.; Reyes, F. E.; Héroux, A.; Batey, R. T. Structural basis for recognition of S-adenosylhomocysteine by riboswitches. *RNA* **2010**, *16*, 2144–2155.

27 Huang, L.; Serganov, A.; Patel, D. J. Structural insights into ligand recognition by a sensing domain of the cooperative glycine riboswitch. *Mol. Cell* **2010**, *40*, 774–786.

28 Stoddard, C. D.; Montange, R. K.; Hennelly, S. P.; Rambo, R. P.; Sanbonmatsu, K. Y.; Batey, R. T. Free state conformational sampling of the SAM-I riboswitch aptamer domain. *Structure* **2010**, *18*, 787–797.

29 Regulski, E. E.; Breaker, R. R. In-line probing analysis of riboswitches. *Methods Mol. Biol.* **2008**, *419*, 53–67.

30 Mortimer, S. A.; Weeks, K. M. A fast-acting reagent for accurate analysis of RNA secondary and tertiary structure by SHAPE chemistry. *J. Am. Chem. Soc.* **2007**, *129*, 4144–4145.

31 Weeks, K. M. Advances in RNA structure analysis by chemical probing. *Curr. Opin. Struct. Biol.* **2010**, *20*, 295–304.

32 Lu, C.; Ding, F.; Chowdhury, A.; Pradhan, V.; Tomsic, J.; Holmes, W. M.; Henkin, T. M.; Ke, A. SAM recognition and conformational switching mechanism in the Bacillus subtilis yitJ S box/SAM-I riboswitch. *J. Mol. Biol.* **2010**, *404*, 803–818.

33 Steen, K. A.; Malhotra, A.; Weeks, K. M. Selective 2′-hydroxyl acylation analyzed by protection from exoribonuclease. *J. Am. Chem. Soc.* **2010**, *132*, 9940–9943.

34 Hampel, K. J.; Tinsley, M. M. Evidence for preorganization of the glmS ribozyme ligand binding pocket. *Biochemistry* **2006**, *45*, 7861–7871.

35 Baird, N. J.; Ferré-D'Amaré, A. R. Idiosyncratically tuned switching behavior of riboswitch aptamer domains revealed by comparative small-angle X-ray scattering analysis. *RNA* **2010**, *16*, 598–609.

36 Lipfert, J.; Sim, A. Y.; Herschlag, D.; Doniach, S. Dissecting electrostatic screening, specific ion binding, and ligand binding in an energetic model for glycine riboswitch folding. *RNA* **2010**, *16*, 708–719.

37 Rambo, R. P.; Tainer, J. A. Improving small-angle X-ray scattering data for structural analyses of the RNA world. *RNA* **2010**, *16*, 638–646.

38 Baird, N. J.; Kulshina, N.; Ferré-D'Amaré, A. R. Riboswitch function: flipping the switch or tuning the dimmer? *RNA Biol.* **2010**, *7*, 328–332.

39 Noeske, J.; Buck, J.; Fürtig, B.; Nasiri, H. R.; Schwalbe, H.; Wöhnert, J. Interplay of "induced fit" and preorganization in the ligand induced folding of the aptamer domain of the guanine binding riboswitch. *Nucleic Acids Res.* **2007**, *35*, 572–583.

40 Noeske, J.; Schwalbe, H.; Wöhnert, J. Metal-ion binding and metal-ion induced folding of the adenine-sensing riboswitch aptamer domain. *Nucleic Acids Res.* **2007**, *35*, 5262–5273.

41 Ottink, O. M.; Rampersad, S. M.; Tessari, M.; Zaman, G. J.; Heus, H. A.; Wijmenga, S. S. Ligand-induced folding of the guanine-sensing riboswitch is controlled by a combined predetermined induced fit mechanism. *RNA* **2007**, *13*, 2202–2212.

42 Buck, J.; Noeske, J.; Wöhnert, J.; Schwalbe, H. Dissecting the influence of Mg^{2+} on 3D architecture and ligand-binding of the guanine-sensing riboswitch aptamer domain. *Nucleic Acids Res.* **2010**, *38*, 4143–4153.

43 Lee, M. K.; Gal, M.; Frydman, L.; Varani, G. Real-time multidimensional NMR follows RNA folding with second resolution. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 9192–9197.

44 Greenleaf, W. J.; Frieda, K. L.; Foster, D. A.; Woodside, M. T.; Block, S. M. Direct observation of hierarchical folding in single riboswitch aptamers. *Science* **2008**, *319*, 630–633.

45 Wilson, R. C.; Smith, A. M.; Fuchs, R. T.; Kleckner, I. R.; Henkin, T. M.; Foster, M. P. Tuning riboswitch regulation through conformational selection. *J. Mol. Biol.* **2010**, *405*, 926–938.

46 Lang, K.; Rieder, R.; Micura, R. Ligand-induced folding of the thiM TPP riboswitch investigated by a structure-based fluorescence spectroscopic approach. *Nucleic Acids Res.* **2007**, *35*, 5370–5378.

47 Rieder, U.; Kreutz, C.; Micura, R. Folding of a transcriptionally acting $preQ_1$ riboswitch. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10804–10809.

48 Rieder, U.; Lang, K.; Kreutz, C.; Polacek, N.; Micura, R. Evidence for pseudoknot formation of class I $preQ_1$ riboswitch aptamers. *ChemBioChem* **2009**, *10*, 1141–1144.

49 Tolić-Nørrelykke, S. F.; Engh, A. M.; Landick, R.; Gelles, J. Diversity in the rates of transcript elongation by single RNA polymerase molecules. *J. Biol. Chem.* **2004**, *279*, 3292–3299.

50 Adelman, K.; La Porta, A.; Santangelo, T. J.; Lis, J. T.; Roberts, J. W.; Wang, M. D. Single molecule analysis of RNA polymerase elongation reveals uniform kinetic behavior. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 13538–13543.

51 Haller, A.; Rieder, U.; Aigner, M.; Blanchard, S. C.; Micura, R. Conformational capture of the SAM-II riboswitch. *Nat. Chem. Biol.* **2011**, *7*, 393–400.

52 Lang, K.; Micura, R. The preparation of site-specifically modified riboswitch domains as an example for enzymatic ligation of chemically synthesized RNA fragments. *Nat. Protoc.* **2008**, *3*, 1457–1466.

53 Rieder, R.; Höbartner, C.; Micura, R. Enzymatic ligation strategies for the preparation of purine riboswitches with site-specific chemical modifications. *Methods Mol. Biol.* **2009**, *540*, 15–24.

54 Micura, R. Small interfering RNAs and their chemical synthesis. *Angew. Chem., Int. Ed.* **2002**, *41*, 2265–2269.

55 Wachowius, F.; Höbartner, C. Chemical RNA modifications for studies of RNA structure and dynamics. *ChemBioChem* **2010**, *11*, 469–480.

56 Höbartner, C.; Rieder, R.; Kreutz, C.; Puffer, B.; Lang, K.; Polonskaia, A.; Serganov, A.; Micura, R. Syntheses of RNAs with up to 100 nucleotides containing site-specific 2′-methylseleno labels for use in X-ray crystallography. *J. Am. Chem. Soc.* **2005**, *127*, 12035–12045.

57 Kloiber, K.; Spitzer, R.; Tollinger, M.; Konrat, R.; Kreutz, C. Probing RNA dynamics via longitudinal exchange and CPMG relaxation dispersion NMR spectroscopy using a sensitive ^{13}C-methyl label. *Nucleic Acids Res.* **2011**, *39*, 4340–4351.

58 Fürtig, B.; Wenter, P.; Reymond, L.; Richter, C.; Pitsch, S.; Schwalbe, H. Conformational dynamics of bistable RNAs studied by time-resolved NMR spectroscopy. *J. Am. Chem. Soc.* **2007**, *129*, 16222–16229.

59 Wenter, P.; Fürtig, B.; Hainard, A.; Schwalbe, H.; Pitsch, S. Kinetics of photoinduced RNA refolding by real-time NMR spectroscopy. *Angew. Chem., Int. Ed.* **2005**, *44*, 2600–2603.

60 Höbartner, C.; Mittendorfer, H.; Breuker, K.; Micura, R. Triggering of RNA secondary structures by a functionalized nucleobase. *Angew. Chem., Int. Ed.* **2004**, *43*, 3922–3925.

61 Corbino, K. A.; Barrick, J. E.; Lim, J.; Welz, R.; Tucker, B. J.; Puskarz, I.; Mandal, M.; Rudnick, N. D.; Breaker, R. R. Evidence for a second class of S-adenosylmethionine riboswitches and other regulatory RNA motifs in alpha-proteobacteria. *Genome Biol.* **2005**, *6*, R70.

62 Brenner, M. D.; Scanlan, M. S.; Nahas, M. K.; Ha, T.; Silverman, S. K. Multivector fluorescence analysis of the xpt guanine riboswitch aptamer domain and the conformational role of guanine. *Biochemistry* **2010**, *49*, 1596–1605.

63 Boehr, D. D.; Nussinov, R.; Wright, P. E. The role of dynamic conformational ensembles in biomolecular recognition. *Nat. Chem. Biol.* **2009**, *5*, 789–796.

64 Leulliot, N.; Varani, G. Current topics in RNA-protein recognition: control of specificity and biological function through induced fit and conformational capture. *Biochemistry* **2001**, *40*, 7947–7956.

65 Duchardt-Ferner, E.; Weigand, J. E.; Ohlenschläger, O.; Schmidtke, S. R.; Suess, B.; Wöhnert, J. Highly modular structure and ligand binding by conformational capture in a minimalistic riboswitch. *Angew. Chem., Int. Ed.* **2010**, *49*, 6216–6219.

66 Stelzer, A. C.; Kratz, J. D.; Zhang, Q.; Al-Hashimi, H. M. RNA dynamics by design: biasing ensembles towards the ligand-bound state. *Angew. Chem., Int. Ed.* **2010**, *49*, 5731–5733.

67 Zhang, Q.; Stelzer, A. C.; Fisher, C. K.; Al-Hashimi, H. M. Visualizing spatially correlated dynamics that directs RNA conformational transitions. *Nature* **2007**, *450*, 1263–1267.

68 Frank, A. T.; Stelzer, A. C.; Al-Hashimi, H. M.; Andricioaei, I. Constructing RNA dynamical ensembles by combining MD and motionally decoupled NMR RDCs: new insights into RNA dynamics and adaptive ligand recognition. *Nucleic Acids Res.* **2009**, *37*, 3670–3679.

69 Hermann, T.; Patel, D. J. Adaptive recognition by nucleic acid aptamers. *Science* **2000**, *287*, 820–825.

70 Heppell, B.; Blouin, S.; Dussault, A. M.; Mulhbacher, J.; Ennifar, E.; Penedo, J. C.; Lafontaine, D. A. Molecular insights into the ligand-controlled organization of the SAM-I riboswitch. *Nat. Chem. Biol.* **2011**, *7*, 384–392.