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Global analysis of riboswitches by small-angle X-ray scattering and calorimetry

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Abstract

Riboswitches are phylogenetically widespread non-coding mRNA domains that directly bind cellular metabolites and regulate transcription, translation, RNA stability or splicing *via* alternative RNA structures modulated by ligand binding. The details of ligand recognition by many riboswitches have been elucidated using X-ray crystallography and NMR. However, the global dynamics of riboswitch-ligand interactions and their thermodynamic driving forces are less understood. By compiling the work of many laboratories investigating riboswitches using small-angle X-ray scattering (SAXS) and isothermal titration calorimetry (ITC), we uncover general trends and common themes. There is a pressing need for community-wide consensus experimental conditions to allow results of riboswitch studies to be compared rigorously. Nonetheless, our meta-analysis reveals considerable diversity in the extent to which ligand binding reorganizes global riboswitch structures. It also demonstrates a wide spectrum of enthalpy-entropy compensation regimes across riboswitches that bind a diverse set of ligands, giving rise to a relatively narrow range of physiologically relevant free energies and ligand affinities. From the strongly entropy-driven binding of glycine to the predominantly enthalpy-driven binding of c-di-GMP to their respective riboswitches, these distinct thermodynamic signatures reflect the versatile strategies employed by RNA to adapt to the chemical natures of diverse ligands. Riboswitches have evolved to use a combination of long-range tertiary interactions, conformational selection, and induced fit to work with distinct ligand structure, charge, and solvation properties.

1. Introduction

Riboswitches are noncoding mRNA domains that control gene expression in *cis*. They are distinguished from other regulatory mRNA domains by their ability to recognize cognate effector molecules directly, without participation of proteins. Although the term “riboswitch” was not used until 2002 [1], the first report of an RNA that satisfies this description was in 1993 [2], and the existence of such regulatory RNA molecules was proposed as early as 1961 [3]. Furthermore, *in vitro* selection experiments have yielded

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numerous RNAs that can recognize ligands without relying on proteins [4]. Some of these “aptamers” have been employed to construct artificial riboswitches [5–7]. Because they are a phylogenetically widespread paradigm of genetic control that regulates key metabolic and signaling pathways, riboswitches have been the subject of numerous genetic, biochemical, biophysical, and structural studies [8–17]. The structural underpinnings for the recognition of ligands by many distinct riboswitch types have been elucidated by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy, as reviewed by Peselis and Segnanov in this special issue. Small-angle X-ray scattering (SAXS) and isothermal titration calorimetry (ITC) are two complementary biophysical techniques that have been extensively employed to characterize riboswitches and their response to ligand binding. SAXS provides low-resolution information on RNA conformation in solution [18], and ITC reports on the enthalpy, entropy and stoichiometry of riboswitch ligand-interactions [19]. Here, we gather the results of many published SAXS (Table 1) and ITC (Table 2) studies on riboswitches, enabling us to perform global meta-analyses. Because of the structural diversity of both riboswitch RNAs and their ligands, the correlations emerging from our compilation suggest general features of RNA-ligand interactions.

2. The application of SAXS to study riboswitch structure and dynamics

In recent years, SAXS has proven itself a valuable tool for RNA research, especially in the study of riboswitches. At a minimum, SAXS provides a measurement of sample homogeneity by detecting the presence of aggregates. For non-aggregated samples, SAXS can measure the radius of gyration (R_g) and the end-to-end distance (D_{max}) of the examined RNA as well as an indication of molecular compactness. The R_g is the square root of the average squared distance of each electron from the center of the molecule and is the parameter focused on below. After relatively straightforward analysis using widely available software (Primus [20], Igor [21], FoXS [22], etc.), SAXS can yield low resolution (~ 20 Å) *ab initio* reconstructions or envelopes that provide basic information about the shape of the molecule or molecular complex in solution [23]. SAXS is particularly suitable for studying nucleic-acid structures thanks to the electron-rich nature of nucleotides compared to amino acids, their non-spherical overall shapes, as well as their prominent secondary structures (RNA helices) exhibiting a typical width of 20–30 Å. Thus, in principle, low-resolution SAXS envelopes of large RNAs can delineate the overall structure and pinpoint principal secondary structural features. This approach is illustrated by the recent structural elucidation of the “A”-shaped HIV-1 Rev response element (RRE) [24]. For a more thorough discussion of SAXS theory, instrument setup, and data analysis, which is beyond the scope of this review, the reader should consult the following excellent reviews [18, 25, 26].

A typical SAXS experiment is conducted on RNA samples over a range of concentrations (e.g., 0.4 – 2 g/L) to ensure that no concentration-dependent phenomena occur, such as dimerization or aggregation. In addition, several exposures are collected to monitor sample radiation damage, which may contribute to aggregation as observed by an increase in R_g as exposure time increases. Scattering data obtained from SAXS experiments are often reported as a plot of momentum transfer q ($\text{\AA}^{-1}$), which is related to the scattering angle, vs. the logarithm of the scattering intensity, I . These data are obtained from the raw scattering data by subtracting buffer scattering, which is significant. Consequently, preparation of

RNA samples often includes purifying folded RNA by size-exclusion chromatography (SEC), which serves to both separate RNA from higher-order oligomers and aggregates and provide a matching SEC buffer sample for background subtraction [27]. Once data have been collected, the R_g may be calculated through Guinier analysis and compared across several RNA concentrations or experimental conditions, such as ligand and Mg^{2+} concentrations as discussed below. The R_g may also be calculated from pair-wise distance distribution function, which is a histogram of intramolecular distances that can be approximated from scattering data. In addition, the compactness (“foldedness”) of the RNA can be deduced from the Kratky plot of $q^2 \cdot I$ as a function of q , which should yield an approximately bell-shaped curve for folded RNAs for $q < 0.2 \text{ \AA}^{-1}$.

SAXS studies of RNAs have often examined the contribution of metal ions such as Mg^{2+} to RNA folding by monitoring R_g or the Kratky plot over a range of Mg^{2+} concentrations [28–30]. In the case of riboswitches, folding is monitored over a range of ligand concentrations as well, following the paradigms of induced fit or conformational capture upon ligand binding by the riboswitch aptamer domain. The choice of conditions in these experiments is critical as the obtained scattering data will be a linear combination of the ideal scattering curves of the “bound” and “free” states. Thus, without a careful titration, the R_g values obtained may not truly represent the R_g values of homogeneous states and only report on the relative compaction. Additionally, a caveat to interpreting R_g values is that two very different RNA conformations may be characterized by the same or similar R_g . For example, the free T-box Stem I has an R_g of 32.7 Å (Table 1), and the riboswitch bound to tRNA^{Gly} has a similar R_g of 31.6 Å, even though the molecular mass of the complex is nearly doubled. This is in part due to Stem I RNA forming an extended helix in solution [31–33]. Thus, the lack of change in R_g is not necessarily consistent with the degree of structural change occurring between the free and bound states.

3. SAXS reveals compaction of some riboswitches induced by ions and ligands

As shown in Table 1, many riboswitches undergo compaction in the presence of Mg^{2+} (as deduced from a decrease in R_g), a phenomenon previously documented for other RNAs such as the *Tetrahymena* ribozyme [30, 34], tRNA^{Phe} and RNase P [29, 35], and the bacterial group I ribozyme [28]. The amount of compaction observed at 10 mM Mg^{2+} is considerable for most riboswitches, with the FMN riboswitch being the exception, having a $R_{g,Mg}$ of $-0.3 \text{ \AA}$ (Table 1), which is within experimental error. In this case, experiments were conducted at 1.5, 2.5, and 10 mM Mg^{2+} , so compaction likely requires less than 1.5 mM Mg^{2+} , and the RNA might be characterized by a more extended state and larger R_g if sub-physiological Mg^{2+} were used [36]. In other cases, however, the degree of compaction upon addition of Mg^{2+} is similar to that provided by ligand binding. For example, the class I c-di-GMP riboswitch shows a $R_{g,Mg}$ of $-3.5 \text{ \AA}$ and an additional decrease in R_g upon ligand binding (Table 1, $R_{g,ligand} = -4.6 \text{ \AA}$). In this case, the relatively large changes in R_g are the consequence of a tetraloop-capped helix pointing away from the center of the riboswitch in the ligand-free state and its inward refolding and binding to a tetraloop receptor in the presence of c-di-GMP (Figure 1A–C) [37]. This reorganization is consistent with the large

positive $-T \Delta S$ for c-di-GMP binding as measured by ITC, one of the largest so far reported for a riboswitch, as discussed below (Table 2). Thus, Mg^{2+} and ligand both play a clear role in the structural reorganization of the c-di-GMP riboswitch, which is unresponsive to c-di-GMP in the absence of Mg^{2+} [37–39].

In contrast to the c-di-GMP riboswitch, the lysine riboswitch undergoes compaction in the presence of Mg^{2+} ($R_{g,\text{Mg}} = -3.9 \text{ \AA}$) but appears unaffected by ligand binding ($R_{g,\text{ligand}} = +0.1 \text{ \AA}$). The lack of response to lysine suggests that either only subtle rearrangements are occurring upon lysine binding or that the binding site is largely preformed in the unbound state. Indeed, the lysine riboswitch crystallized in the presence and absence of lysine exhibits largely the same structure aside from the absence of a potassium ion that coordinates the bound lysine [40]. The TPP and SAM-I riboswitches both exhibit strong compaction in the presence of Mg^{2+} and more modest compaction in the presence of ligand (Fig. 1D–F & Table 1) [36, 41, 42]. For the *Vibrio cholerae* glycine riboswitch and the SAM-III/ S_{MK} riboswitch, relatively large $R_{g,\text{ligand}}$ of $-8 \text{ \AA}$ and $-4.5 \text{ \AA}$ suggest more pronounced structural rearrangement upon ligand binding. Like the SAM-II riboswitch, SAM-III is short and characterized by one of the smallest R_g among the RNAs in Table 2. In the crystal structure of SAM-III, the ligand is bound in a bulge formed at the junction of three stems, one of which comprises only two base pairs and is closed by a tetraloop [43]. The ligand-free state is likely to be partially unfolded, which is consistent with the lack of imino peaks in NMR spectra at 25°C , relative to the SAM-III-bound RNA [44] and the large $R_{g,\text{ligand}}$. The large $R_{g,\text{ligand}}$ for the *V. cholerae* glycine riboswitch was also observed in glycine riboswitches from other bacteria [45]. Glycine binding has been predicted to facilitate interdomain contacts between the two domains of the riboswitch [45, 46], but precisely how glycine binding drives this reorganization is unknown.

Overall, riboswitches vary greatly in their response to Mg^{2+} and ligand as measured by SAXS. In general, their R_g values correlate well with their length even in the unbound state (Fig. 2A, $R = 0.96$). The RNA constructs examined in Table 1 are in part selected for monodispersity as those constructs were often used for structural studies demanding homogeneity. Extended or unfolded RNAs would be generally characterized by R_g values above the regression lines in Fig. 2A for a given length [36, 42, 44]. For example, the ~ 100 nt TARPolyA domain of the HIV-1 genome is characterized by an R_g of $38 \text{ \AA}$, and the reconstructed molecular envelopes are consistent with two helices pointing in opposite directions from one another [47]. Like their proteinaceous ligand-binding counterparts, riboswitches frequently adopt a relatively compact structure to assemble a binding pocket, often approximating the compactness of tRNAs (Fig. 2A).

To a first approximation, it might be expected that the extent to which riboswitches compact upon ligand binding (as monitored as a SAXS, $R_{g,\text{ligand}}$) might correlate with the total entropy, if global compaction is the primary source of the binding entropy. However, the riboswitches studied to date do not exhibit such a correlation (Fig. 2B, $R = 0.44$), suggesting that the entropic term is dominated by factors other than global structural compaction, such as ligand desolvation and localized conformational changes, neither of which are detected by SAXS measurements.

4. Calorimetric studies of riboswitches produce full thermodynamic profiles associated with ligand binding

SAXS data are informative on riboswitch global conformation but are not amenable to model-free thermodynamic analysis. The improvement in commercial calorimeters, particularly the increased sensitivity and stability of microcalorimeters that require only few hundred microliters of sample, make calorimetry the method of choice for measuring small molecule binding to proteins, DNA, and more recently, riboswitches [48, 49]. Differential Scanning Calorimetry (DSC) and ITC are the two most commonly used calorimetric approaches. Both use a power compensation system to monitor the amount of instrument power required to maintain a constant temperature of the sample cell *versus* a reference cell. The difference is that the temperature of both cells in DSC is slowly raised until a melting transition is triggered, whereas in ITC instrument power is fed into the cells to compensate for the heat evolved or absorbed due to the binding reaction, so that the temperature remains constant across both cells [48]. DSC and ITC directly measure the reaction enthalpy change and can produce complete thermodynamic profiles of binding including stoichiometry n , enthalpy change H , entropy change S , Gibbs free energy change G , and heat capacity change C_p [50, 51]. Recently, data analysis strategies to also extract kinetic parameters such as k_{on} and k_{off} by fitting heat profiles of individual ITC titration injections have been developed, further expanding the utility of ITC in bimolecular association studies [52, 53].

5. Local and global sources of enthalpy and entropy changes

Binding of a specific ligand to its cognate riboswitch aptamer domain can be expected to reorganize the local structure of the binding site, as well as to induce global conformational changes of varying magnitude (Fig. 1) [36, 42, 44]. Overall ligand-induced compaction of the aptamer domain, as evidenced by a reduction in R_g measured by SAXS (Table 1) has been documented for many riboswitches but is not a universal feature [36, 42, 44]. For some riboswitches, crystallographic or NMR structures have been reported for both the ligand-free and -bound states, revealing varying degrees of structural reorganization. Therefore, enthalpy and entropy changes that accompany ligand binding to riboswitches can be expected to report on local and global structural changes, formation of new tertiary interactions, and changes in quaternary structure, in addition to changes in solvation and counterion distribution, as well as other molecular properties, such as vibrational modes.

A survey of riboswitch ITC data in the literature reveals appreciable variation in the conditions under which measurements were taken (Table 2). Given the dependence of thermodynamic parameters on temperature, pH, concentration of Mg^{2+} , ionic strength, etc, caution is needed when comparing the results of experiments performed under different conditions in different laboratories.

The enthalpy of ligand binding by different riboswitches varies from strongly endothermic to strongly exothermic (Table 2). Upon complex formation, polar groups from both the ligand and the riboswitch exchange hydrogen bond partners from water to each other. The newly formed hydrogen bonds, when optimal in distance and geometry, can yield a favorable enthalpy of as much as -5 kcal/mol per bond [49]. On the other hand, unfavorable

enthalpy associated with desolvation opposes the hydrogen bond exchange and ligand binding. In addition to hydrogen bonds, van der Waals interactions, generally dictated by shape complementarity of the ligand to the binding site, can produce additional favorable enthalpy. For the glycine riboswitch, the unfavorable enthalpy observed in glycine binding to either a single or tandem glycine aptamer RNA ($\sim +6$ kcal/mol [45]) is likely explained by the high cost of desolvating the polar ammonium and carboxylate groups (as high as $+8$ kcal/mol per group [54]) compared to the modest favorable enthalpy gained from weak hydrogen bonds from glycine binding to RNA bases, which is assisted by Mg^{2+} ions [46]. Because glycine lacks a side chain, van der Waals contacts between glycine and the RNA are minimal, leading to a net unfavorable enthalpy of binding. In contrast, c-di-GMP binding to its cognate riboswitch substantially remodels the RNA architecture (Fig. 1A–C) and triggers formation of additional long-range tertiary contacts between a tetraloop and a tetraloop receptor, which could contribute up to -10 kcal/mol of favorable enthalpy [52]. Such new bond formation is manifest as enthalpic gain in addition to that from ligand binding, leading to a large net enthalpy change of -42 kcal/mol [37, 38].

The entropy changes associated with ligand binding have some recognizable local and global structural origins. Complex formation between ligand and RNA invariably suffers an entropy penalty arising from reduction of the translational and rotational degrees of freedom of the two molecules and partial immobilization of otherwise rotatable bonds, but also gains favorable entropy from release of water molecules and counterions from the ligand and its binding site on the RNA. With nucleic acids and proteins that significantly change conformation upon ligand binding, there is additional, almost always unfavorable, entropy associated with immobilizing flexible RNA helices or protein domains through tertiary interactions. For instance, in the case of the class I c-di-GMP riboswitch, the entropic cost of forming the tetraloop-receptor interactions upon ligand binding may account for much of the total opposing entropy term of $+31$ kcal/mol [37, 38]. Comparison of tRNA binding to the T-box riboswitch stem I (nucleotide 14–113, 100 residues) versus the longer 14–158 fragment (145 residues, which encompasses the stem I, the part of the antiterminator that base-pairs with tRNA 3' end and a linker) shows that the longer T-box RNA exhibits higher favorable enthalpy (-41 versus -25 kcal/mol) but also higher opposing entropy ($+32$ versus $+16$ kcal/mol) [33]. This thermodynamic signature is consistent with the formation of additional base pairs between the tRNA 3' end and the longer T-box RNA, and a concomitant entropic cost arising from partially immobilizing a flexible linker region. Collectively, the enthalpic gain and entropic loss neutralize each other, leading to comparable affinities towards tRNA (“entropy-enthalpy compensation” is discussed below) [33].

6. Differences between *in vitro* selected aptamers and natural riboswitches

Comparison of the thermodynamic profiles of twelve representative riboswitches and one *in vitro* selected aptamer (Table 2, Fig. 3) reveal that ligand binding to these RNAs is primary driven by favorable enthalpy changes (mean $H = -22$ kcal/mol) and is partially impeded by unfavorable entropy changes (mean $-T S = 12$ kcal/mol), producing a mean net Gibbs free energy change of approximately -8 ± 3 kcal/mol, which translates into an average ligand dissociation constant of $1.1 \mu M$ at $20^\circ C$. This mean affinity exhibited by riboswitches likely

reflects the intracellular regulatory requirements for a typical small molecule metabolite. A previous meta-analysis of the contributions of ligand burial by *in vitro* selected aptamers and natural riboswitches towards their overall binding affinity (or Gibbs free energy) showed a strong linear correlation ($R = 0.89$) [55]. However, the correlation was much stronger for *in vitro* selected aptamers while the natural riboswitches (only six were available then) showed no significant correlation. Now, with the expanded set of twelve naturally occurring riboswitches, it is clear that unlike the artificial aptamers, natural riboswitches exhibit only weak correlation between the ligand solvent-accessible surface buried and the binding free energy G ($R = 0.50$), enthalpy H ($R = 0.52$), or entropy $-T \Delta S$ ($R = 0.55$). This curious distinction between natural riboswitches and artificial aptamers could stem from their different sizes as well as their distinct evolutionary origins. *In vitro* selected aptamers are selected and amplified in the laboratory only to achieve the desired property as dictated by the selection criterion, that is, binding to the ligand [4]. The lengths of the aptamers are limited by technical capabilities of DNA synthesizers and library complexity, and usually intentionally kept short, to increase coverage of sequence space and reduce undesired RNA-RNA interactions. The resulting aptamers usually exhibit simple folds and are rarely sufficiently long and structurally complex to exhibit substantial long-range (tertiary) interactions. Thus, the thermodynamic response to ligand binding by aptamers is usually dominated by local changes at and near the binding site (or the degree of global ligand-induced structural changes is similar across different types of *in vitro* selected aptamers). It follows that, the ligand solvent-accessible area buried is strongly correlated with the binding free energy, on theoretical grounds [56]. In contrast, natural riboswitches have been subject to millions (or billions) of years of evolution under much more diverse selective pressures to achieve biologically relevant affinities, appropriate co-transcriptional folding and binding kinetics, as well as high selectivity in the presence of structurally similar competing ligands. This has led to the frequent use of long-range tertiary and even quaternary interactions [57, 58]. It is noteworthy that ligand-induced global conformational changes commonly observed for natural riboswitches (reported by changes in R_g) also correlate only weakly with thermodynamic parameters of binding such as the total entropy change (Fig. 2B, $R = 0.44$). This suggests that the conformational entropy associated with global riboswitch conformational changes does not dominate the total entropy change either. Thus, unlike the dominant contribution of localized ligand-RNA interactions to *in vitro* selected aptamer binding, the thermodynamic profiles of natural riboswitches arise from both, localized ligand-RNA interactions, and global conformational changes. It would be of interest to replace natural riboswitch aptamers with their *in vitro* selected counterparts of similar affinity to ask if the natural riboswitches are functionally superior in the cellular milieu.

7. Parsing enthalpic and entropic contributions illuminate ligand binding mechanisms

Mechanistic insights can be gleaned about the process of small molecule association with macromolecules by parsing the total thermodynamic contributions into enthalpic and entropic components [48, 49]. Previous analyses of small molecules binding to DNA and proteins have revealed a balancing act between the two components [48, 49].

Two classes of small molecules bind duplex DNA through very different mechanisms. “Groove binders”, such as Hoechst 33258, do not alter the conformation of the DNA duplex and exhibit binding driven by favorable entropy and minimal, and often unfavorable, enthalpy. In contrast, “intercalators”, such as ethidium bromide, force reorganization of DNA stacking in order to accommodate the intercalators, and rely on very large favorable enthalpy changes to offset the cost associated with formation of distorted DNA structures [48]. Application of this analysis to ligand binding by riboswitch RNAs makes evident a strong linear anti-correlation between H and $-T \Delta S$ ($R = 0.98$), consistent with the notion that physiologically relevant ligand affinity can be achieved by a wide range of enthalpic and entropic combinations. At one end of the spectrum is the glycine riboswitch, to which glycine binds with a small, unfavorable enthalpic change that is compensated by favorable entropic changes [45, 46]. Similar to DNA groove binders, glycine binds a largely preformed binding site on one of the tandem aptamers and exhibits minimal heat change, presumably due to its small size and limited hydrogen-bonding capabilities [45, 46]. On the opposite end of the spectrum are the c-di-GMP and guanine riboswitches binding to c-di-GMP and hypoxanthine, respectively. Binding of c-di-GMP is driven by a very large favorable enthalpic change (-42 kcal/mol) that offsets the unfavorable entropic cost ($+32$ kcal/mol). Somewhat analogous to DNA intercalators, c-di-GMP binding significantly remodels the RNA architecture and triggers formation of additional long-range tertiary contacts between a tetraloop and a tetraloop-receptor (Fig. 1A–C) [37, 38]. Such new bond formation is energetically manifest as additional enthalpic gain beyond ligand binding and partly offsets the entropic cost of immobilizing otherwise mobile RNA helices. This interpretation is supported by crystallographic, SAXS, and single-molecule FRET analyses [37, 38].

While the anti-correlation of enthalpy and entropy exhibited by ligand binding to riboswitches is striking, the theoretical grounds and generality of “enthalpy-entropy compensation” are a subject of active debate [59, 60]. Enthalpy and entropy are not fundamental quantities but are two intertwined terms that are a function of the microscopic configurations of the system, including the ligand, the macromolecule, and the surrounding solvent and counterions [59]. Several models attempt to explain the widely observed enthalpy-entropy compensation in bimolecular interactions among small molecules, DNA, and proteins. One simple notion is that enthalpy-entropy compensation arises because of the intrinsic properties of the hydrogen bond—energetically more favorable hydrogen bonds are usually more restrictive in distance, angle, and bond rotation, thus usually are accompanied by unfavorable entropy [61–63]. Other ideas focus on the effect of solvent reorganization and propose that compensation might be a fundamental property of all processes occurring in water [64]. It has been noted that the optimal measurement range of calorimetry instruments, frequently larger than reported errors in enthalpy measurements, and publication bias in reporting well-behaved, physiologically relevant affinities, may have contributed to the “window” effect that may have exaggerated the universality of enthalpy-entropy compensation [59].

Historically, researchers in different fields have used “enthalpy-entropy compensation” in different ways. Some have applied this concept fairly narrowly, in considering a series of

otherwise identical reactions investigating the response of the system to a singular perturbation, e.g., temperature, pH, etc[65, 66]. Investigators in drug-protein and drug-DNA interactions have observed this compensation phenomenon among binding reactions between a given macromolecule and a panel of related compounds, an analysis that has produced significant insights to optimize drug leads [48, 61]. Further work has suggested empirical compensation occurring among similar binding reactions involving unrelated ligands binding to dissimilar macromolecules, such as ligand binding to DNA [48] and site-specific DNA-binding proteins binding to their DNA targets [67]. Our meta-analysis suggests that “enthalpy-entropy compensation” can be extended to the discussion of ligands binding to structured RNAs such as riboswitches. It is perhaps not surprising that ligand binding reactions to proteins, DNA and RNA all exhibit enthalpy-entropy correlations as these interactions are mediated by the same types of physical forces and occur in the same solvent.

8. Multiple, structurally distinct ligand-free states?

A minimalist view of riboswitch function postulates the existence of two conformational states: free and ligand-bound. However, studies of several riboswitches support the notion that the free state of riboswitches represents an ensemble of two or more structurally distinct states that interconvert, influenced by temperature and divalent ion concentrations [38, 44, 68–73].

Structured RNAs have a tendency to form dynamic, meta-stable structures that may facilitate their interaction with other molecules. The central portion of the stem B of the hairpin ribozyme undergoes a dramatic rearrangement (RMSD = 6.0 Å) when docking with stem A to form the active site for transesterification, which involves the extrahelical extrusion of two uridine bases [68, 74]. Interestingly, a NMR study of the isolated stem B revealed a minor but appreciable set of nuclear Overhauser effect (NOE) cross peaks that are characteristic of the docked structure [69]. Thus the stem B in isolation already exists in a presumably dynamic equilibrium between the free and docked structures, and the approaching stem A selects the minor, docked stem B structure to form the active site [68]. NMR and ITC analysis of the SAM-II riboswitch (or S_{MK}) suggested that in the absence of SAM, the RNA exists in equilibrium between a binding-competent, “primed” conformation, and a binding-incompetent conformation [44]. Another NMR study, of the *add* adenine riboswitch, reveals that this RNA exists in two distinct conformations that are in an equilibrium that is shifted by temperature, thus compensating for the temperature effects on adenine affinity for the RNA [70]. Similar findings were also reported of the c-di-GMP riboswitch using single-molecule Fluorescence Resonance Energy Transfer (FRET) analysis, which revealed that the free riboswitch populates four distinct states, which include two static populations of “extended” and “docked” riboswitches that do not respond to ligand binding, as well as two dynamic populations that undergo conformational changes in response to ligand binding but are preferentially “extended” and “docked”, respectively [38]. The distribution of the RNA among the four populations is shifted by Mg^{2+} and ligand binding, and can, in physiological Mg^{2+} and saturating c-di-GMP concentrations, reach a distribution dominated by the static, docked state [38].

It is conceivable that such three-state and multi-state mechanisms may also add additional layers of regulation, allowing integration of cellular signals such as osmolarity change with ligand concentration (and temperature) to operate these genetic switches. This is reminiscent of the *B. subtilis glmS* riboswitch, which has been shown to integrate inhibitory and activating inputs from different hexose metabolites for refined control of cellular metabolism [75]. The existence of conformational partition of ligand-free aptamers into binding-competent and binding-incompetent fractions has already been postulated based on unexpected observations of temperature-dependent changes in heat capacity [72]. The next challenge is to obtain definitive *in vivo* evidence that the conformational partition of the ligand-free riboswitches occurs to similar extent in the cell and is not a result of RNA misfolding induced by *in vitro* manipulations such as denaturation and non-physiological ion and buffer conditions.

9. Conformational selection, induced fit, or both?

The existence of pre-organized ligand-free conformations that are poised for rapid ligand engagement seems to support the conformational selection view for ligand binding to riboswitches [44, 70, 73]. The act of ligand binding then depletes this population and draws the equilibrium towards the formation of more molecules in ligand binding-competent conformations. However, the thermodynamic signature of enthalpy-driven DNA intercalators, DNA aptamers and the c-di-GMP and guanine riboswitches suggests the existence of an induced fit mechanism as well. Indeed, ligand binding by twelve out of thirteen riboswitches analyzed in Fig. 3D is enthalpically driven, most likely employing induced fit. Induced fit is implied by crystallographic analysis of tRNA association with the T-box riboswitch stem I, as both the tRNA and the T-box stem I must undergo global and local conformational changes before shape complementarity can be achieved [33]. There is no reason *a priori* that conformational selection and induced fit have to be mutually exclusive. The ligand-free RNA can sample a number of structures including a singular or several binding-competent states (Fig. 4). However, none of the binding-competent states are necessarily structurally identical to the ligand-bound state. For instance, key tertiary contacts may have already formed in these intermediate states but the binding site for the ligand may not have formed or only partially formed (Fig. 4). Ligand binding can then further induce a significant local or even global restructuring of the binding-competent intermediate to arrive at the *bona fide* energetic minimum.

10. Future prospects

In the literature on the thermodynamic analysis of ligand-riboswitch interactions, the contributions of solvent are generally not explicitly parsed from those of the ligand and macromolecule. These contributions, in particular the entropic changes associated with the reconfiguration of the water network upon ligand binding and accommodation, could be very significant. New methodological developments especially in NMR, may allow more straightforward measurement of solvent contributions and these may refine existing descriptions of the structure-thermodynamics relationships in many biological systems.

Many riboswitches, in particular those that modulate gene expression at the transcriptional level, have a strong kinetic component in their regulatory mechanisms [12, 76, 77]. The speed and other kinetic characteristics of the cognate RNA polymerase, the distribution and duration of the transcriptional pauses, RNA folding kinetics and thermodynamics all contribute to the switching function. Few riboswitches have been studied by SAXS or ITC with experimental RNA constructs that encompass both the metabolite-sensing aptamer domains and the expression platforms that interface with the rest of the gene expression machinery. There is a further paucity of studies that examine riboswitch function in their native, functional context, e.g., as it emerges from the RNA polymerase and folds co-transcriptionally. Finally, we propose that a standard set of conditions (e.g., 20 °C, pH 7.0, 2.5 mM Mg²⁺, 100 mM KCl) under which future SAXS and ITC experiments should be performed, in addition to other conditions that the experimenter may favor, in order to permit comparison between results from different laboratories and enable more rigorous global analyses of riboswitch-ligand interactions.

With an increasing understanding of the structural, kinetic, and thermodynamic profiles of ligand binding to aptamers and riboswitches, the field is poised to address how ligand recognition is coupled to genetic switching, and how the observed dynamic range and switching behavior fit the regulatory needs of the cell.

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Highlights

- Meta-analyses of SAXS and ITC data on riboswitch-ligand interaction.
- Riboswitches exhibit a wide spectrum of enthalpy-entropy compensation regimes.
- Neither local nor long-range interactions dominate thermodynamics.
- Conformational selection and induced fit coexist in riboswitch function.

Fig. 1.

Structural contexts of cyclic-di-GMP and thiamine pyrophosphate (TPP) binding to their respective cognate riboswitches. (A) Crystal structure of cyclic-di-GMP bound to its riboswitch (3IWN). (B) SAXS-derived molecular envelope of the cyclic-di-GMP riboswitch bound to its cognate ligand in 2.5 mM Mg^{2+} , superimposed on the crystal structure in (A). (C) SAXS-derived molecular envelope of the ligand-free cyclic-di-GMP riboswitch in 2.5 mM Mg^{2+} . (D) Crystallographic analysis reveals that TPP binds across two parallel RNA helical stacks and stabilizes a compact riboswitch conformation (PDB: 2HOJ). (E) SAXS-derived molecular envelope of the TPP riboswitch bound to TPP in 2.5 mM Mg^{2+} , superimposed on the crystal structure in (D). (F) SAXS-derived molecular envelope of the ligand-free TPP riboswitch in 2.5 mM Mg^{2+} .

Fig. 2.

Global SAXS analysis of riboswitches. (A) The radius of gyration (R_g) of riboswitches in solution correlates linearly with RNA length. Riboswitch data for RNAs in the free (hollow, blue squares) and ligand-bound (filled, black circles) states in the presence of Mg^{2+} is from Table 2 and references therein. Correlation analysis yields a Pearson's R value of 0.96 for ligand-free riboswitches and 0.93 for ligand-bound riboswitches. Data for tRNA^{Phe} is shown for comparison and is from [27]. (B) Entropy of ligand binding versus the extent of riboswitch compaction (R_g). Poor correlation ($R = 0.44$) suggests that the entropy change that accompanies ligand binding is not dominated by the conformational entropy arising from global reorganization.

Fig. 3.

Calorimetric analysis of riboswitches. (A–C) Solvent-accessible ligand surface area buried (Å²) by riboswitch binding does not correlate strongly with the binding enthalpy (A), free energy (B), or entropy (C). This contrasts with the strong correlation ($R = 0.89$) between binding free energy and solvent-accessible area buried by *in vitro* selected aptamers [55]. (D) A wide spectrum of entropy-enthalpy distributions demonstrate significant compensation ($R = 0.98$), resulting in a relatively narrow range of ligand binding affinities for riboswitches.

Fig. 4.

Dynamic, multi-step mechanisms of ligand binding to riboswitches. Ligand-free riboswitches can exist in two or more structurally and dynamically different forms. A conformational equilibrium between binding-competent and binding-incompetent structures can be modulated by temperature, Mg²⁺ concentration, etc. This allows the riboswitch to sample a variety of distinct structures, some of which are primed to engage the ligand rapidly. Attaining the native, ligand-bound state may also require induced fit to form a ligand-binding site that does not pre-exist in any of the ligand-free structures.

Table 1SAXS-derived R_g values for riboswitches in the presence and absence of ligands

Ligand	State	R_g (Å)	$R_{g,ligand}^a$	$R_{g,Mg}^b$	Length	Ref.
FMN	Free	29.4	-0.5	-0.3	141	[36]
	Bound	27.9				
	Free + 10 mM Mg^{2+}	29.1				
	Bound + 10 mM Mg^{2+}	28.6				
c-di-GMP	Free	32	-4.6	-3.5	98	[37]
	Free + 2.5 mM Mg^{2+}	28.5				
	Bound + 2.5 mM Mg^{2+}	23.9				
	Free	27.5	-2.3	-3.1	83	[36]
TPP	Bound	24.1				
	Free + 10 mM Mg^{2+}	24.4				
	Bound + 10 mM Mg^{2+}	22.1				
	Free	29.9	-1.1	-2.2	94	[36]
SAM-I	Bound	26.3				
	Free + 10 mM Mg^{2+}	27.5				
	Bound + 10 mM Mg^{2+}	26.4				
	Free	43	0.1	-3.9	181	[36]
Lysine	Bound	43.3				
	Free + 10 mM Mg^{2+}	39.1				
	Bound + 10 mM Mg^{2+}	39.2				
	Free + 10 mM Mg^{2+}	43	-8	n/a		[45]
Glycine ^c	Bound + 10 mM Mg^{2+}	35				
	Free	31.7	-0.8	-10	52	[78]
	Bound	21.5				
	Free + 10 mM Mg^{2+}	20.7				
SAM-II	Bound + 10 mM Mg^{2+}	19.5				

Ligand	State	R_g (Å)	$R_{g, \text{ligand}}^a$	$R_{g, \text{Mg}}^b$	Length	Ref.
SAM-III/S _{MK}	Free + 5 mM Mg ²⁺	25	-4.5	n/a	51	[44]
	Bound + 5 mM Mg ²⁺	20.5				
tRNA ^{Gly}	Free + 15 mM Mg ²⁺	32.7	-1.1	n/a	86/161 ^d	[31]
	Bound + 15 mM Mg ²⁺	31.6				

^a Difference in R_g between samples in the ligand-bound and ligand-free states, both in the presence of Mg²⁺.

^b Difference in R_g between the free samples in the presence and absence of Mg²⁺.

^c Glycine riboswitch from *Vibrio cholera* is reported.

^d T-box Stem I is 86 nt, and the complex in the presence of tRNA^{Gly} is 161 nt.

Table 2

Thermodynamic and kinetic parameters for ligand-riboswitch interactions

Ligand/substrate	Riboswitch or ribozyme	H (kcal mol ⁻¹)	$-T \Delta S$ (kcal mol ⁻¹)	k_{on} (10 ⁴ M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	K_d (nM)	Solvent-accessible area buried (Å ²)	PDB accession code	Ref.
Adenine	<i>B. subtilis</i> pbuE	n/a*	n/a	26	0.15	581	n/a	1Y26	[76]
Adenosylcobalamin (AdoCbl)	<i>E. coli</i> htrB Cobalamin riboswitch	n/a	n/a	n/a	n/a	250	n/a	n/a	[79]
Cyclic-di-GMP	<i>V. cholerae</i> tfoX	-42	31 ^a	1.7	1.8x10 ⁻⁷	0.011	650	3WVN	[38, 80]
7-deazaG	<i>Streptococcus mutans</i> THF riboswitch	-30	24 ^c	n/a	n/a	38000	n/a	n/a	[81]
Fluoride (KF)	<i>Thermotoga petrophila</i> fluoride riboswitch	-3.3	1.5 ^e	n/a	n/a	2.8x10 ⁵	190	4ENC	[82]
FMN	<i>B. subtilis</i> ribD	-15	6 ^b	20	0.002	12	549	3F2Q	[36, 77]
Glycine	<i>B. subtilis</i> tandem Glycine riboswitch (with Kink-turn)	6	-12 ^e	n/a	n/a	15000	199	3OWI	[45, 46]
Guanosine	<i>Tetrahymena</i> group I ribozyme	n/a	n/a	0.7	0.77	1.1x10 ⁵	n/a	1X8W	[83]
Hypoxanthine	<i>B. subtilis</i> xpt-pbuX	-35	28 ^a	n/a	n/a	3000	267	4FE5	[84]
Lysine	<i>C. acetobutylicum</i>	-23	15 ^b	n/a	n/a	1800	380	3DIL	[36]
Methylcobalamin (MeCbl)	<i>E. coli</i> htrB Cobalamin riboswitch	n/a	n/a	n/a	n/a	15000	n/a	n/a	[79]
PreQ ₁ -I	<i>F. nucleatum</i>	n/a	n/a	60	0.17	283	305	3FU2	[85]
SAH	<i>Ralstonia solanacearum</i> SAH riboswitch	-21	12 ^a	n/a	n/a	90	400	3NPQ	[86]
SAM-I	<i>T. tengcongensis</i> metF-methH2	-20	12 ^b	n/a	n/a	1350	715	2GIS	[36]
SAM-II	<i>Sargasso Sea metagenome</i> MetX SAM-II riboswitch	~ -22	~18 ^a	n/a	n/a	670	656	2QWY	[87]
SAM-III (S _{MR})	<i>E. faecalis</i> metK SAM-III riboswitch	-19	9 ^d	n/a	n/a	63	605	3E5C	[44]
Tetracycline	<i>In vitro</i> selected aptamer	-23	11 ^c	n/a	n/a	0.8	423	3EGZ	[88]
Theophylline	<i>In vitro</i> selected aptamer	n/a	n/a	17	0.07	300	n/a	1EHT	[89]

Ligand/substrate	Riboswitch or ribozyme	H (kcal mol ⁻¹)	$-T \Delta S$ (kcal mol ⁻¹)	k_{on} (10 ⁴ M ⁻¹ s ⁻¹)	k_{off} (s ⁻¹)	K_d (nM)	Solvent-accessible area buried (Å ²)	PDB accession code	Ref.
TPP	<i>E. coli</i> thiM	-24	15 ^a	8.7	0.043	198	490	2H0J	[80, 85]
tRNA ^{Gly}	<i>B. subtilis</i> GlyQS T-box Stem I (14–113)	-25	16 ^e	n/a	n/a	150	802	4LCK	[33]
tRNA ^{Gly}	<i>B. subtilis</i> GlyQS T-box 14–158	-41	32 ^e	n/a	n/a	194	n/a	n/a	[33]

* n/a, not available or not included.

^a $T = 303$ K,

^b $T = 310$ K,

^c $T = 298$ K,

^d $T = 288$ K,

^e $T = 293$ K