**The Metabolome Weakens RNA Helix Stability and Increases RNA Chemical Stability**

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ABSTRACT: Herein, we examine the complex network of interactions among RNA, the metabolome, and divalent Mg2+ in conditions that mimic the *E. coli* cytoplasm. We determined Mg2+ binding constants for the top 15 *E. coli* metabolites, comprising 80% of the total metabolome, at physiological pH and monovalent ion concentrations. These data were used to inform development of an artificial cytoplasm that mimics *in vivo E. coli* conditions, termed “Eco80”. We empirically determined that the mixture of *E. coli* metabolites in Eco80 approximates single site binding behavior towards Mg2+ in the biologically relevant free Mg2+ range of ~0.5 to 3 mM Mg2+, using a Mg2+ sensitive fluorescent dye. Effects of Eco80 conditions on the thermodynamic stability, chemical stability, structure, and catalysis of RNA were examined. We find that Eco80conditions lead to opposing effects on the thermodynamics and chemical stability of RNA. In particular, the thermodynamic stability of RNA helices was weakened while the chemical stability and catalysis of RNA were enhanced, which can be understood using the speciation of Mg2+ between weak and strong Mg2+-metabolite complexes in Eco80. Overall, the effects of Eco80 reflects RNA function *in vivo* and enhances the biological relevance of mechanistic studies of RNA *in-vitro*.

**Introduction**

RNA serves as the conduit of genetic information in the Central Dogma of Molecular Biology and performs numerous essential functions in cellular biology owing in part to the capacity of RNA to form complicated, diverse, and functional structures.1 The development of genome wide structure-probing techniques *in vivo* has provided valuable insight into RNA structure and function cells.2–4 However, most experimental techniques that provide valuable insight into the mechanism and function of RNA cannot be readily performed in a cell, and are typically limited to simple conditions, usually 100 to 500 mM monovalent metal ions and 0.5 to 50 mM free divalent magnesium ions (Mg2+) with a dilute buffer.5 I*n vitro* studies of RNA in conditions that mimic a cell, so called *in vivo*-like conditions, provide a valuable link between experiments that probe RNA structure *in vivo* and experiments that provide biologically-relevant mechanistic and thermodynamic insight *in* *vitro*.5

Many studies have investigated the effects of individual components of the cellular environment on nucleic acid structure, including small molecules and abiological crowders. Studies that use small molecules that are similar to metabolites in cells indicate that these species interact strongly with the unfolded state of nucleic acids, destabilizing RNA and DNA secondary structure.1–5 Studies that simulate the cellular crowding effects of macromolecules reveal stabilized RNA tertiary structures, increased folding cooperativity, and improved RNA function.6–10 Thermodynamic characterization of RNA helix formation in crowding conditions indicates that crowders destabilize helices and that the nearest neighbor model for calculating RNA structure applies in conditions that mimic the cell.11–13 In summary, using simple models to simulate elements of the cellular environment *in vitro* has provided valuable mechanistic insight into how the cell affects nucleic acid structure and motivated investigation of more complex and realistic artificial cytoplasms.14

A number of studies have performed mechanistic studies of proteins in extremely complex environments,15 ranging from cell lysates to live cells.16–18 Such top down approaches to complexity provide valuable insight into how proteins behave in the cell. However, researchers sacrifice control over the environment that is provided by a simple system. Mechanistic studies of RNA in the cell or lysate have two additional problems. The first is the propensity of cells to degrade foreign RNA.19,20 The second is the lack of control of Mg2+ speciation between free and chelated Mg2+ in lysate because a cell will cease to regulate Mg2+ concentrations once it is lysed.

Control over Mg2+ speciation is crucial for mechanistic studies of RNA because of the sensitivity of RNA folding and function to the concentration of Mg2+ in the solution, as demonstrated by thousands of studies.21 Furthermore, recent studies have demonstrated the importance of weak metabolite-Mg2+ complexes to RNA function.22–24 These studies consider effects of mixtures of one to three metabolites, which is a step forward but still far from the true complexity of the cellular environment. In addition, Mg2+ speciation is approximate in these studies, as Mg2+ speciation was estimated assuming single-site-binding, meaning that one metabolite interacts with one Mg2+ ion, and binding constants are extrapolated from published sources, which typically are reported at disparate ionic compositions and pHs.21

Herein, we take a bottom up, *aufbau*, approach that builds up complexity to make an artificial cytoplasm that contains 80% of *E. coli* metabolites, with biologically relevant concentrations of monovalent ions and free Mg2+ ions.We start by compiling metabolite concentrations in *E.* coli, simplify to the 15 most abundant metabolites, determine metabolite-Mg2+ binding constants at the relevant pH and ionic strength, and lastly determine how much total Mg2+ concentration is present in the final mixture of metabolites. This *aufbau* approach allows us to understand the effects of the metabolite and metal ion species that comprise a major part of the network of interactions that RNA experiences in *E. coli* cells.

**Results**

**Eco80: An artificial cytoplasm containing 80% of *E. coli* metabolites**

*E. coli* cells contain hundreds of metabolites (~240 mM total), 25 which is too many to test systematically for Mg2+ binding and RNA stability. However, the 15 most abundant metabolites in *E. coli*, an experimentally manageable number, comprise 80% (195 mM) of total metabolites (Figure 1A). We thus sought to prepare Eco80*,* an artificial cytoplasm containing biological concentrations of the 15 most abundant metabolites in *E. coli* (Table 1).

Eco80 was prepared at a 2x concentration so that it could be diluted into other reagents and contain physiological concentrations of monovalent metal ions at pH 7.0 (see supplementary information (SI) table 1 for details). Briefly, all metabolites in Eco80 are zwitterions or negatively charged near physiological pH 7, which requires electrostatic neutralization with metal ions. Metabolite salts and free acids were prepared to a final 2xconcentration, and the amount of Na+ and K+ added with each metabolite was recorded. Next, the pH of the 2xstock was adjusted to pH 7.0 using NaOH, and the amount of Na+ was recorded. Lastly, NaCl and KCl were added to a final 480 mM Na+ and 280 mM K+, twice the physiological value of 240 mM Na+ and 140 mM K+. The 2x concentrated artificial cytoplasm was then diluted into other reagents to a final 1x concentration for experiments.

Next, we considered how metabolites affect the speciation of free and chelated Mg2+. All 15 Eco80 metabolites have functional groups, carboxylates and phosphates, that drive chelating interactions with Mg2+ ions (Table 1), and we have previously estimated thatthe metabolite pool in *E. coli* has potential to chelate 51 mM Mg2+, assuming 2 mM free Mg2+, at an ionic strength of 0.15 M and a pH of 7.5.21 While extensive literature exists on chelating interactions between Mg2+ and small molecules, our previous estimates are approximate as Mg2+ binding affinity is dependent on environmental factors such as pH, ionic strength, composition of background ions, and temperature.26–32 Thus, we sought to more precisely characterize Mg2+ chelation by the metabolites inEco80,at the physiological background.

We determined apparent disassociation constants (*KD*) for Eco80 metabolites in a background of 240 mM NaCl, 140 mM KCl, pH 7.0 buffer at 37 °C (Table 1). Isothermal titration calorimetry (ITC) was used to measure the *KD*s for phosphorylated metabolites (SI figure 1, SI table 2). A fluorescence assay, which measures the free Mg2+ concentration in a sample using the metal ion-binding dye 8-Hydroxy-5-quinolinesulfonic (HQS) acid,33 was used to estimate the *KD* for Mg2+ for metabolites that did not produce enough heat on binding Mg2+ to measure with ITC (SI figure 2, SI table 3). For this assay, Mg2+ is titrated into HQS solutions in the absence and presence of weak to strong Mg2+ chelators. Emission of HQS as a function of the total Mg2+ in the absence of chelators is then fit to a binding model for the binding of Mg2+ to HQS (Equation 1) (SI figure 2A, top black data and fit). The free Mg2+ concentration, which is equal to the total Mg2+ concentration in the absence of chelator, is then associated with the fluorescence emission for each data point using the binding model. This process is repeated in the presence of chelator, using the no-chelator data to obtain free Mg2+ concentration at any total concentration of Mg2+. Note that free and total Mg2+ concentrations are the same, y=x, in the absence of chelators, and that the data are right-shifted in the presence of chelators (SI figure 2A, bottom). The affinity of Mg2+ binding by metabolites is thus obtained by fitting the free Mg2+ concentration as a function of the total Mg2+ concentration (SI equation 4).

The binding affinity for Eco80 metabolites and Mg2+ ranged from strong to negligible. The four nucleotide triphosphates, ATP, UTP, GTP, and dTTP, were classified as strong Mg2+ binders, with *KD* values ranging from 0.160 to 0.28 mM, less than the approximate free Mg2+ concentration in *E. coli* of 2 mM (Table 1). Conversely, 8 other metabolites--L-glutamic acid, fructose 1,6-BP, UDP-N-acetylglucosamine, glucose 6-phosphate, L-aspartic acid, 6-phospho-gluconic acid pyruvic acid, and dihydroxyacetone phosphate--were classified as weak Mg2+ binders with *KD* values greater than 2 mM (Table 1). Three metabolites--glutathione, L-valine, and L-glutamine--had negligible Mg2+ binding properties, as measured with HQS (SI figure 2). In an effort to understand the effects of Eco80 on RNA mechanistically, we created two sub-artificial cytoplasms: NTP-chelated Mg2+ (NTPCM) and weak metabolite-chelated Mg2+ (WMCM), comprised of the strong Mg2+ chelators (NTPs) and weak Mg2+ chelators, respectively (Table 1).

We used two methods to estimate how Eco80 metabolites affect the speciation of free and chelated Mg2+. The first method was the same HQS assay that we used to estimate binding constants for metabolites that did not produce enough heat on Mg2+ binding to measure with ITC, based on calculating the free Mg2+ concentration in the presence of metabolites using HQS fluorescence emission (Figure 1B-D SI table 4). This method directly determines free Mg2+ but does not report on speciation of Mg2+ to different metabolites. The second method used a statistical model that accounts for experimental uncertainty in metabolite concentrations and uncertainty in *KD* determination, and estimates Mg2+ speciation assuming single-site binding (meaning that one metabolite associates one Mg2+ ion). The second method approximates Mg2+ speciation to different metabolites but does not account for non single-site metabolite Mg2+ interactions and does not directly determine free Mg2+ concentration. The statistical model is described in detail in the Supplementary Methods. Briefly, concentration errors were propagated from uncertainties in reagent masses and volumes used during sample preparation, and *KD* uncertainties were obtained from the fits (Table 1). Both uncertainties were then randomly seeded into Equation 1 1000 times to create the same virtual artificial cytoplasm with different errors, where [Mg]T is the total Mg2+ concentration, [Mg] is the free Mg2+ concentration, “*i"* is an integer representing each metabolite in a mixture, N is the total number of metabolites in a mixture, [Li]T is the concentration of the “*i’th*” metabolite in a mixture, and *KD* is the dissociation constant.

Then, equation 1 was solved numerically to determine the free Mg2+ concentration produced at a given total Mg2+ concentration in a virtual artificial cytoplasm.

On the basis of agreement of the HQS data and the statistical simulation, methods 1 and 2, respectively, the two methods support a model in which Mg2+ speciates in artificial cytoplasms largely according to a single-site model within or below the biological free Mg2+ concentration range of 0.5 to 3 mM Mg2+. However, Mg2+ does not speciate according to a single-site model at higher free Mg2+ concentrations (Figure 1 E-G).

In Eco80, the statistical model suggests that the metabolites should buffer the free Mg2+ concentration in the biological Mg2+ range, where a 20 mM increase in the total Mg2+ from 20 to 40 mM leads to only a 2.5 mM increase in free Mg2+ from 0.5 to 3 mM (Figure 1E, hex bins). Free Mg2+ concentrations measured in this range with HQS emission are consistent with this single-site behavior (Figure 1E, black data points). At higher free Mg2+ concentrations, Eco80 should lose its free Mg2+ buffering capacity as chelators become saturated, and the free Mg2+ should increase with the total Mg2+ (model in Figure 1E, hex bins). However, the free Mg2+ concentration measured with HQS does not increase as fast as the statistical model predicts above 3 mM free Mg2+ (Figure 1E, compare black data points and hex bins). For example, free Mg2+ in Eco80 is expected to increase from 3 mM to ~100 mM as the total Mg2+ concentration is increased from 40 mM to 200 mM (Figure 1E, hex bins). However, the free Mg2+ concentration measured with HQS only increases from 3 mM to ~10 mM (Figure 1E, data points). One possibility is that multivalent interactions, which the statistical model does not account for, where several Mg2+-saturated metabolites interact with additional Mg2+ molecules, dominate the equilibrium. Such non-single-site behavior above 3 mM free Mg2+ is also observed in the NTPCM and WMCM artificial cytoplasms (Figure 1 F & G), and was observed previously.21

Lastly, we sought to empirically determine how much total Mg2+ is required to attain a free Mg2+ concentration of 2 mM in Eco80, NTPCM, and WMCM. The relationship between the free Mg2+ calculated from HQS emission and the total Mg2+ concentration in each artificial cytoplasms was fit to a polynomial to empirically approximate the data (Figure 1 E-G, blank lines), and the total Mg2+ concentration required to produce 2 mM Free Mg2+ was calculated from the polynomial fit (see methods for details). This resulted in predicted 31.6, 25.0, and 6.5 mM total Mg2+ concentration to produce 2 mM free Mg2+ in Eco80, NTPCM, and WMCM, respectively (Table 2).

**Thermodynamic analysis of RNA helices in Eco80 by fluorescence binding isotherms**

We sought to understand how Eco80 affects the thermodynamic stability of RNA. We began our study on RNA helices comprised of Watson-Crick base pairs. Stability of RNA helices has been traditionally measured with UV-absorbance-detected melting curves, typically monitored at 260 or 280 nm.34,35 However, such absorbance melting curves are not appropriate for measuring helix stability in Eco80 because of the high absorptivity of the nucleotide metabolites--ATP, UTP, GTP, dTTP, and UDP-N-acetylglucosamine--which are major components of Eco80. Thus, we pursued a fluorescence-detected binding isotherm assay, which is optically orthogonal to Eco80.

Helix stability was monitored using the emission of a 5’-fluorophore-labeled RNA strand (FAM-RNA) in equilibrium with a complementary 3’-quencher labeled RNA strand (RNA-BHQ1) (Figure 2A). High emission indicates that the FAM-RNA is single-stranded, while low emission indicates that it is bound in duplex with an RNA-BHQ1 strand. We used a binding isotherm method, where RNA-BHQ1 is titrated into a constant concentration of FAM-RNA at several temperatures (SI figure 3), resulting in a series of apparent binding isotherms (Figure 2B). We favored FDBIs over fluorescence-detected melts because of the severe dependence of FAM emission on temperature.36–38 Emission of FAM was monitored in a Real-Time PCR instrument at different temperatures, resulting in a isotherm every 0.5 °C from 20 to 80 °C (Figure 2B). See supplemental methods for details.

Raw fluorescence was fit with MeltR, a new program created by the authors, to determine folding energies. MeltR is a package of functions in the popular R programming language that allows facile conversion of raw data to folding energies (see Methods for details). Importantly, MeltR handles two sources of experimental error, uncertainty in RNA concentration and inaccurate *KD*s collected at low and high temperatures. MeltR handles uncertainties in RNA concentration by using a Job plot obtained from fluorescence isotherms at low temperatures. Likewise, MeltR allows the user to select isotherms that provide the most accurate *KD*s, which are then passed to the non-linear regression algorithms that determine helix folding energies. MeltR then calculates folding energies using two Van’t Hoff methods: (1) directly fitting a Van’t Hoff plot as a function of temperature (Figure 3C) and (2) globally fitting raw fluorescence emission to SI equation X (SI table 5). Errors in the main text are reported 1.5% in terms of the ΔG°37 and a detailed error analysis is in the methods. In the next three sections we examine the effect of Eco80 on the thermodynamic stability, chemical stability, and catalysis of representative RNA sequences.

**Eco80 thermodynamically destabilizes RNA helices**

We used fluorescence binding isotherms to determine helix folding energies in background monovalent metal ion control (240 mM NaCl 140 mM KCl) with either Eco80, NTPCM, and WMCM for a set of five representative eight base-pair RNA helices; all solutions contain 2 mM free Mg2+, as per Table 2. This helix set was designed to contain representatives of all 10 Watson-Crick nearest neighbor parameters and vary in AU content from 25% to 75%. Both of the aforementioned methods to determine folding energies in MeltR agreed (SI table 5) so the results from the Van’t Hoff plot were reported. Results are summarized in Table 3, which is ranked according to the AU content of the duplex.

All five representative helices were significantly destabilized in Eco80 relative to the background monovalent condition, meaning the ΔΔG°37 between the background monovalent condition and Eco80 was larger than its propagated uncertainty (Table 3, Figure 2D). We did not observe a clear relationship between AU content and destabilization. For example, Helix 2 has a relatively low AU content of 38% and was strongly destabilized by 1.1±0.3 kcal/mol in Eco80. However, helix 1 and 5, the sequences with the lowest (25%) and highest (75%) AU content respectively, were destabilized by equal amounts, 0.6±0.3 and 0.6±0.2 kcal/mol, respectively. Thus, Eco80 destabilizes RNA helices but the underlying sequence dependence is not apparent.

To better understand how the various components of Eco80 contribute to destabilizing RNA helices, we analyzed the effects of the strong and weak Mg2+-chelating metabolites separately. NTPCM, which is comprised of strong Mg2+-chelating metabolites, consistently destabilized RNA helices (Figure 2D), by 0.32 to 0.60 kcal/mol (Table 3). The destabilizing effect of Eco80 appears to be related to the AU content of the helix with destabilization increasing linearly from +0.32 kcal/mol at 25% AU content to 0.60 kcal/mol at 75% AU content /%(R2 = 0.99, SI figure 4).

In contrast, WMCM, which is composed of weak Mg2+-chelating metabolites, destabilized, had no effect, or stabilized RNA helices in a fashion that did not depend on AU content (Figure 2D, Table 3). Similar to Eco80, the sequence dependence of stabilization or destabilization is not clear. Overall, the net effect of Eco80 on RNA helices is destabilization, with AU-content-dependent destabilizing interactions dominating for strong Mg2+ chelating metabolites, and a mixture of stabilizing and destabilizing interactions for weak Mg2+ chelating metabolites. Apparently weak Mg2+ chelation gives rise to a hidden sequence dependence that carries over to Eco80 (see Discussion).

**Eco80 protects RNA from chemical degradation**

Several studies indicate that weak and strong Mg2+ chelating metabolites reduce Mg2+-mediated RNA degradation22,39 To assess whether Eco80 stabilizes the chemical structure of RNA, we used an in-line probing (ILP) assay, which takes advantage of the natural susceptibility of the RNA phosphodiester backbone to cleavage.40 In this mechanism, the 2’-hydroxyl is deprotonated by a Mg2+-hydroxide (Mg2+-OH-), and the 2’-hydroxyl serves as a nucleophile to attack the adjacent phosphate in an SN2-like mechanism (Figure 3A). Unstructured nucleotides are more susceptible to cleavage because they are more likely to adopt an in-line conformation that favors cleavage.40 For this assay, 5’-32P RNAs are incubated at 37 °C for about 90 h to facilitate in-line cleavage, with time points taken regularly. RNA fragments are then fractionated on a denaturing PAGE gel (SI figure 5), providing single nucleotide resolution of degradation rates measured by the increase in counts with time for a given band provided by a linear fit. In-line degradation rates for biological RNA in Eco80, NTPCM, and WMCM with enough total Mg2+ to maintain 2 mM free Mg2+ were compared to degradation rates in a 2 mM free Mg2+ and 25 mM free Mg2+ condition. The 25 mM free Mg2+ condition was chosen because it is a common free Mg2+ condition *in-vitro* and is similar to the 25 and 31.6 total Mg2+ condition used for NTPCM and Eco80, respectively (Table 2).

We first determined in-line degradation rates for the guanine riboswitch aptamer with different artificial cytoplasms (Figure 3B). The guanine riboswitch aptamer has been studied extensively, thus providing structural and mechanistic information.41–43. We chose to study the guanine riboswitch in its guanine ligand unbound, apo, state for experimental simplicity. The expression platform was trimmed to prevent structural switching and the guanine ligand was not added to the solution to favor the apo state. Moreover, guanine binding to the aptamer induces structural changes only at nucleotides directly mediating the guanine binding site, 41 indicating that structural information provided by X-ray crystal structures of the ligand bound aptamer is relevant for a structural analysis of degradation rates.

Great care was taken in our analysis to confirm that the guanine aptamer adopted a similar structure between conditions. The guanine riboswitch aptamer exhibited similar degradation patterns between the 2 mM free, Eco80, NTPCM, and WMCM conditions, with high degradation in the 5’-region of the P2 stem and high reactivity in the P3-stem loop region (L3), indicating that the apo guanine riboswitch aptamer adopts a similar structure in these conditions (Figure 3C, SI figure 6). The 25 mM free Mg2+ condition exhibited higher degradation rates than the other conditions in the J2/3 junction (Figure 3C). This pattern is similar to ILP data published for another guanine riboswitch at a higher pH and Mg2+ concentration,44 indicating that the increase in degradation rates in the 25 mM free Mg2+ condition is dependent on the presence of Mg2+-OH- complexes (SI figure 6). To further confirm that the guanine aptamer adopts similar structures between all conditions, we collected small angle X-ray scattering (SAXS) data on the apo form of the aptamer in every artificial cytoplasm. Raw scattering profiles overlay on another, indicating that the structure of the guanine aptamer is similar between conditions (SI figure 7A). Guinier analysis and p(r) analysis, where the maximum is the radius of gyration (SI figure 7B), reveal similar radius of gyration between solution conditions (SI table 6). Lastly, electron density reconstructions and bead model reconstructions are consistent with the crystal structure of the guanine riboswitch aptamer in every condition (SI figure 7C-G). Thus, decreased degradation rates in the 2 mM free, Eco80, NTPCM, and WMCM conditions in comparison to the 25 mM free condition is likely due to a reduction in the availability Mg2+-OH- complexes caused by chelation of Mg2+ by metabolites rather than large changes in RNA structure.

We sought to better characterize the structural dependence of structured RNA degradation in different conditions. We therefore extended the study to the CPEB3 ribozyme and tRNAPhe (SI figure 8, 9, & 10). We inspected the crystal structure of for these two RNAs plus the original guanine aptamer and manually classified each residue by manual inspection, as single stranded (SS), meaning that the base was not participating in hydrogen bonding interactions with other residues, non-canonical (NC), meaning that the base was forming non-canonical hydrogen bonding interactions with other residues in the tertiary structure, and Watson-Crick (WC), meaning that the base was in a helix composed mostly of Watson-Crick base pairs (SI table 7). Rates of ILP were then analyzed in box plots (Figure 3D-F).

We begin box plot analysis with the guanine aptamer (Figure 3D). We had data for 3 single stranded nucleotides without accompanying non-canonical hydrogen bonding interactions. We observed decreased degradation rates at the single stranded (SS) nucleotides in 2 mM free Mg2+, Eco80, NTPCM, and WMCM, in comparison to the 25 mM free Mg2+ condition. Likewise, we observed an overall decrease in reactivity in 2 mM free Mg2+, Eco80, NTPCM, and WMCM in comparison to the 25 mM free Mg2+ condition for nucleotides involved in non-canonical tertiary interactions (NC). In contrast, degradation rates for nucleotides participating in Watson-Crick base pairing interactions were independent of solution conditions. Thus, we observed a trend of protection from Mg2+ mediated degradation in flexible regions that are susceptible to Mg2+OH- mediated hydrolysis by artificial cytoplasm, even with similar amounts of total Mg2+ in solution as the 25 mM free Mg2+ condition.

We repeated our in-line degradation assay with the cleaved-CPEB3 ribozyme and yeast tRNAphe, to test whether the reduction of Mg2+ mediated degradation in artificial cytoplasm was broadly applicable (SI figure 8, 9, & 10). For the cleaved-CPEB3 ribozyme, degradation rates at single-stranded residues were reduced in 2 mM free Mg2+, Eco80, and NTPCM conditions in comparison to the 25 mM free Mg2+ condition (Figure 3E). Interestingly, the degradation rates of single-stranded residues recovered in WMCM, indicating a that degradation rates are partially dependent on the strength of Mg2+ chelation. Likewise, the degradation rates for residues that are predicted to participate in a non-canonical tertiary contacts were reduced in 2 mM free Mg2+, Eco80, and NTPCM but not WMCM, in comparison to the 25 mM free Mg2+ condition, further indicating a that degradation rates are partially dependent on the strength of Mg2+ chelation. Degradation rates were similar for nucleotides participating in Watson-Crick base-pairs between all conditions.

Yeast tRNAphe exhibits almost no in-line degradation except for the single stranded nucleotides in the P3 stem loop, termed the anticodon loop (SI figure 9D), consistent with published data on well folded tRNAphe. Single stranded nucleotides, mostly from the anticodon loop showed reduced degradation rates in the in the 2 mM free Mg2+, Eco80, and NTPCM conditions in comparison to the 25 mM free condition, and degradation rates recovered in WMCM. Degradation rates were constant across conditions for nucleotides that form Watson-Crick base pairs and for nucleotides that form non-canonical contacts, which is different than the increased degradation observed for nucleotides that form non-canonical base pairs in the guanine riboswitch aptamer and the CPEB3 ribozyme. One possible explanation is that the tertiary structure of tRNAphe is less dynamic than the tertiary structure of the guanine riboswitch aptamer and the CPEB3 ribozyme, thus reducing the degradation rates in regions that participate in non-canonical tertiary interactions to the baseline levels observed for nucleotides participating in Watson-Crick base pairs in 25 mM Mg2+.

Thus, overall, the in-line degradation assay indicated that Eco80 and NTPCM protect RNA from Mg2+OH--mediated degradation in structural regions that are susceptible to in-line cleavage, even though all both artificial cytoplasm’s have relatively high total concentrations of total Mg2+. WMCM shows an intermediate effect between the high degradation rates in susceptible regions exhibited the 25 mM free Mg2+ condition and the low degradation rates in susceptible regions exhibited by 2 mM free Mg2+, Eco80, and NTPCM, indicating that degradation rates are dependent on Mg2+ chelation strength (see Discussion).

**Eco80 supports RNA catalysis**

Weak metabolite-chelated Mg2+ is known to promote catalysis by ribozymes. For example, CPEB3 ribozyme catalysis is enhanced by about 1.6-fold by 2 mM free Mg2+ in solution with an estimated 11.3 mM free glutamate-chelated Mg2+, in comparison to catalysis in 2 mM free Mg2+ alone.22 . Thus, we also hypothesized that Eco80 metabolites would also promote CPEB3 catalysis.

We compared CPEB3 ribozyme cleavage rates in 2 mM free Mg2+ and 25 mM free Mg2+ to Eco80, NTPCM, and WMCM containing enough total Mg2+ to produce 2 mM free Mg2+ (Table 2). Briefly, we purified full length CPEB3 ribozyme (Figure 4A), incubated CPEB3 in artificial cytoplasm’s, fractionated cleaved and un-cleaved CPEB3 from time points on a denaturing acrylamide gel, and calculated the fraction cleaved from the relative intensity of cleaved and un-cleaved RNA bands (SI Figure 11). Fraction cleaved as a function of time was fit to a single exponential equation to estimate the reaction rate constant (Figure 4B). We performed 4 technical repeats per condition.

CPEB3 ribozyme catalysis was reduced in all conditions in comparison to the 25 mM free Mg2+ control (Figure 4C). Surprisingly, CPEB3 catalysis was reduced in Eco80 by about 1/2 in comparison to the 2 mM free Mg2+ control, despite the 31.6 mM total Mg2+ in Eco80. CPEB3 catalysis was reduced by about 1/3 in NTPCM in comparison to the 2 mM free Mg2+ control, an even stronger effect than Eco80. In contrast, CPEB3 catalysis was enhanced by 1.33 fold in WMCM, similar to the enhancement observed for glutamate-chelated Mg2+. In summary, Eco80 supports RNA catalysis but does not enhance catalysis in comparison to the 2 mM free Mg2+ condition. Interestingly, CPEB3 reaction rates in Eco80 are between the rates in WMCM and NTPCM. WMCM likely has exposed Mg2+ to help fold the RNA, while NTPCM does not, and moreover may denature the RNA as per Figure 2 (see Discussion).

WMCM may be more biologically relevant for studying CPEB3 ribozyme function than Eco80. We performed the same analysis of absolute metabolite concentrations in yeast and mammalian imbk cells, which have a closer evolutionary relationship to human cells, as the analysis we used to prepare Eco80 (Figure 4D). Absolute metabolite concentrations were compiled from the literature and the 11 most abundant metabolites that compose 80% of the Yeast and mammalian metabolome were selected to compose hypothetical Yeast80 and Mammal80 artificial cytoplasms.45 Estimated metabolite/Mg2+ binding constants21 were used to classify each metabolite in Yeast80 and Mammal80 as a strong (NTP) or a weak Mg2+ chelator. We found that Yeast80 and Mammal80 would be depleted in strongly chelated-Mg2+, with Yeast80 having no strong Mg2+ chelators and Mammal80 having ~4 mM strong Mg2+ chelators. (Figure 4D). Thus, the 1.3-fold rate enhancement in WMCM in comparison to 2 mM free Mg2+ alone is likely more relevant to CPEB3 function in human cells than the rate decrease in Eco80.

**Discussion**

Paragraph 1 Making Eco80

In summary, we have used a bottom-up, *aufbau*, approach to create a complex but manageable artificial cytoplasm, termed Eco80, which encapsulates 80% of the *E. coli* metabolome (Figure 5A). We also broke down Eco80 into sub-artificial cytoplasms, which contain either metabolites that strongly chelate Mg2+ (NTPs), or metabolites that weakly chelate Mg2+, providing mechanistic insight into the effects of Mg2+ speciation on RNA in cells.

Paragraph 2 HQS as a tool for understanding Mg2+ speciation.

Importantly, we have adopted the Mg2+ sensitive dye, HQS,33 to measure Mg2+ speciation in artificial cytoplasms. A key challenge to studying RNA in *in vivo*-like conditions is knowing how the components the researcher is adding will affect the speciation of Mg2+ between free and chelated Mg2+ species. Published Mg2+ binding constants for cellular components are unreliable, as they apply to solutions with specific ionic character.26 More often, published Mg2+ binding constants do not exist for cellular macromolecules and crowders.21 Lastly, predicting Mg2+ speciation using binding constants require making assumptions about the stoichiometry of Mg2+-component complexes, which may or may not be valid. For example, in this work, the free Mg2+ concentration in Eco80, NTPCM, and WMCM approximated the free Mg2+ concentration that was predicted using binding constants and assuming one-to-one stoichiometry in the biological range of 0.5 to 3 mM free Mg2+, but the prediction was not accurate at higher free Mg2+ concentrations (Figure 1 E-G). Thus, the HQS assay provides information on Mg2+ speciation in messy, biologically relevant solutions, without requiring assumptions or accurate Mg2+ binding constants. Although we used this assay to directly measure Mg2+ speciation in mixtures of metabolites, this assay can applied to other biological molecules.

Paragraph 3 Free Mg2+ buffering in the cell

Our Mg2+ speciation calculations and HQS experiments indicate that metabolites play an important role in buffering the free Mg2+ concentration in cells. Recent theoretical and experimental work has demonstrated that the cellular environment buffers the concentration of biological molecules, thus reducing concentration noise *in* *vivo*.46,47 Single-site-Mg2+ interactions in Eco80 buffer the free Mg2+ concentration between 0.5 and 3 mM Mg2+ given a total Mg2+ change of 20 mM. This buffering effect is exaggerated at even higher total Mg2+ concentrations in Eco80, likely driven by multivalent Mg2+ interactions with metabolites, where an increase in the total Mg2+ concentration to 200 mM only increases the free Mg2+ concentration to ~10 mM.

Paragraph 4 Model that balances interactions that cause opposing effects (Figure 5)

Our thermodynamic analysis of RNA helices in Eco80 indicates that the *E. coli* metabolome has a net destabilizing effect on RNA helices, with destabilizing effects dominating for NTP-chelated Mg2+ and a mixture of destabilizing and stabilizing effects observed for weak metabolite-chelated Mg2+ (Figure 2D). The effects of our artificial cytoplasm on RNA helix stability can be understood using a model that combines the well-established effects of polar small molecules and Mg2+ on RNA secondary structure stability. Polar small molecules are known to interact favorably with the exposed polar bases of nucleic acids in the unfolded state (Figure 5B).48–51 Likewise, Mg2+ is known to interact favorable with the high density of negative charge in helical RNA. Thus, metabolites increase helix formation energies (destabilizes) by favoring the unfolded state and Mg2+ increases (stabilizes) helix formation energy by favoring the helical state (Figure 5C). The changes in helix formation energy Mg2+/metabolite mixtures demonstrate a balance between metabolites favoring the unfolded state and Mg2+ favoring the helical state (Figure 5C). For example, NTPCM strongly chelates Mg2+, thus sequestering Mg2+ from interacting with the folded state so that the destabilizing interactions between NTPs and RNA dominate, leading to a consistent destabilization of RNA helices (Figure 2D). In contrast, WMCM only weakly sequesters Mg2+ so that Mg2+ is available for favorable interactions with helices. Thus, WMCM stabilizes RNA helices when the stabilizing interactions between Mg2+ and the helical state are larger than the destabilizing interactions between Mg2+ and the unstructured state, WMCM destabilizes RNA helices when the stabilizing interactions between Mg2+ and the helical state are smaller than the destabilizing interactions between Mg2+ and the unstructured state, and WMCM leads to no change in helix formation energies when the two effects balance. This leads to the inconsistent destabilization/stabilization of RNA helices observed in WMCM (Figure 2D).

Paragraph 5 Effects on helices, NTPS alone

NTPCM destabilizes AU rich helices more than GC rich helices (SI figure 4). A similar destabilizing effect on G-quadruplex structure has been observed for cytidine nucleotides.52 Interestingly, other nucleotides (A and C) had a much less significant destabilizing effect, indicating that G-quadruplexs are destabilized by favorable base-pairing interactions between cytidine nucleotides in solution and Gs in the unfolded state of the RNA. NTPCM is mostly composed of ATP, UTP, and dTTP (22.5 mM total versus 4.9 mM GTP). ATP, UTP, and dTTP are expected to form stronger hydrogen bonds with As and Us in the unfolded state of RNA, explaining the AU dependence of helix destabilization by NTPCM (SI figure 4).

Paragraph 6 Effect on backbone

Our analysis of RNA degradation rates in Eco80 indicates that metabolites protect regions of RNA that are susceptible to in-line cleaved from Mg2+-OH- mediated degradation (Figure 3). Eco80 and NTPCM had the strongest protective effects, while WMCM had an intermediate protective effect, indicating that protection from Mg2+-OH- mediated degradation is dependent on the strength of the chelating interaction between metabolites and Mg2+. In this model, in-line cleavage of the RNA backbone is limited by the formation of Mg2+-OH- species, which is favorable for free Mg2+, unfavorable for weakly chelated Mg2+, and negligible for strong NTP-chelated Mg2+ (Figure 5D). Thus, RNA degradation reaction rates are weakly reduced by depletion of active Mg2+-OH- species in the presence of weak Mg2+ chelators and degradation reaction rates are strongly reduced by depletion of active Mg2+-OH- species in the presence of strong Mg2+ chelators (Figure 5E).

Paragraph 7 Effect on catalysis

Our analysis of CPEB3 catalysis in Eco80 indicates that cellularly relevant metabolite-Mg2+ mixtures support RNA catalysis. A previous study of hammerhead ribozyme catalysis in the presence of nucleotides found that reaction rates were enhanced by NDP chelated-Mg2+, a weakly-chelated Mg2+ species, and that NTP-chelated Mg2+ has no effect on reaction rates.23 Similarly, our results in metabolite mixtures found that WMCM enhanced CPEB3 ribozyme catalysis while NTPCM inhibited CPEB3 ribozyme catalysis. A previous study of CPEB3 ribozyme in the presence of weak amino acid-chelated Mg2+ indicated that rate enhancement was not driven by direct interactions between ammino acid-chelated Mg2+ and the catalytic site, but by stabilization of catalytically relevant CPEB3 ribozyme structure.22 Likewise, the thermodynamic destabilization of helices observed in Eco80 and NTPCM indicate that reduction CPEB3 catalysis is caused by destabilization of the catalytically relevant structure. Thus, ribozyme rate enhancement *in vivo* is likely dependent on the presence of weak metabolite chelators that stabilize the catalytically relevant structure and depletion of strong chelators that destabilize the catalytically relevant structure.

Paragraph 8 Conclusion

Eco80 has opposing effects on RNA which reflect the complexity of the cellular environment. The thermodynamic stability of RNA helices was weakened by Eco80, the chemical stability of RNA was enhanced by Eco80, and the catalysis of RNA was supported by Eco80. These seemingly contradictory effects can be understood the speciation of Mg2+ between weak and strong Mg2+-metabolite complexes in the biologically relevant metabolite mixture. Overall, the effects of Eco80 on RNA reflects RNA function *in vivo* and enhances the biological relevance of mechanistic studies of RNA *in vitro*.

**Diagram

Description automatically generated with medium confidenceFigure 1** Analysis of Mg2+ speciation in *E. coli* metabolitemixtures. **(A)** *E. coli* metabolome molar composition. Eco80 contains the 15 most abundant metabolites that comprise 80% of the *E. coli* metabolome. NTPCM contains four strong Mg2+ chelating NTPs, and WMCM contains 11 other weak Mg2+ binding metabolites. **(B-D)** Effect of Mg2+ on 8-hydroxyquinoline-5-sulphonic acid (HQS) emission with and without mixtures of metabolites that chelate Mg2+. Grey lines represent fits to determine the binding constant for Mg2+ and HQS in the absence of chelators. **(E-G)** Relationship between the total Mg2+ concentration and the free Mg2+ concentration with mixtures of metabolites that chelate Mg2+. Hex bins represent 1000 statistical simulations of the virtual artificial cytoplasms based on experimental errors in *KD* determination, experimental errors in reagent concentrations, and single-site binding. Triangle data points are free Mg2+ concentrations calculated using HQS emission. Black lines were generated using polynomial regression. The red shaded region is the biological free Mg2+ concentration zone of 0.5 to 3 mM. The red line is the approximate free Mg2+ concentration in *E. coli* of 2 mM.

Table 1. Eco80: The 15 most abundant metabolites, which comprise 80% of the *E. coli* metabolome.

|  |  |  |  |
| --- | --- | --- | --- |
| Metabolite | Conc. (mM)a | *KD*  (mM) | Chelation strengthe |
| ATP | 9.63 (0.963) | 0.28 (0.01)b | NTPCM (Strong) |
| UTP | 8.29  (0.829) | 0.248 (0.004)b | NTPCM (Strong) |
| GTP | 4.87  (0.487) | 0.201  (0.007)b | NTPCM (Strong) |
| dTTP | 4.62  (0.462) | 0.160  (0.003)b | NTPCM (Strong) |
| L-Glutamic acid | 96  (9.6) | 520 (50)c | WMCM (Weak) |
| Glutathione | 16.6  (1.66) | NAd | WMCM (Weak) |
| Fructose 1,6-bisphosphate | 15.2  (1.52) | 5.9 (0.1)b | WMCM (Weak) |
| UDP-N-acytylglucosamine | 9.24  (0.924) | 29 (2)b | WMCM (Weak) |
| Glucose 6-phosphate | 7.88  (0.788) | 17.3 (0.2)b | WMCM (Weak) |
| L-Aspartic acid | 4.23  (0.423) | 465 (12)c | WMCM (Weak) |
| L-Valine | 4.02  (0.402) | NAd | WMCM (Weak) |
| L-Glutamine | 3.81  (0.381) | NAd | WMCM (Weak) |
| 6-Phospho- gluconic acid | 3.77  (0.377) | 14.4 (0.2)b | WMCM (Weak) |
| Pyruvic acid | 3.66  (0.366) | 15.8 (0.9)c | WMCM (Weak) |
| Dihydroxyacetone phosphate | 3.06  (0.306) | 20 (1)b | WMCM (Weak) |

aUncertainty propagated from uncertainties in reagent masses and volumes used during sample preparation. Extra significant digits included to avoid systematic rounding errors in the statistical model. bDetermined at 37 °C with Isothermal titration calorimetry as measured in SI figure 1 and SI table 2. Error is the propagated standard error in the fit parameters. cDetermined at 37 °C with HQS emission as measured in the SI figure 2 and SI table 3. Error is the propagated standard error in the fit parameters. dNo binding observed as per SI Figure 2. eMetabolites with *KD*s for Mg2+ less than 2 mM are considered strong Mg2+ chelators and *KD*s greater than 2 mM are considered weak Mg2+ chelators. NTPCM and WMCM are sub-artificial cytoplasms composing Eco80, a nucleotide triphosphate-chelated Mg2+ and weak metabolite-chelated Mg2+, respectively.

Table 2. Total Mg2+ concentrations used to obtain 2 mM free Mg2+ in artificial cytoplasm.

|  |  |  |  |
| --- | --- | --- | --- |
| Condition | [Total Mg2+] (mM) | [Chelated Mg2+] (mM) | [Free Mg2+] (mM) |
| Eco80 | 31.6 | 29.6 | 2.0 |
| NTPCM | 25.0 | 23.0 | 2.0 |
| WMCM | 6.4 | 4.5 | 2.0 |

Chart

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**Figure 2** *E. coli* metabolite Mg2+ mixtures destabilize RNA secondary structure. **(A)** Layout of a fluorescence binding isotherm assay in a Real-Time PCR machine. **(B)** Raw fluorescence binding isotherms fit to determine equilibrium constants with MeltR. Data points represent raw data. Curves represent curve fits. Colors represent different temperatures (purple: 32.3, blue: 41.8, teal: 51.3, green: 54.6, yellow: 58.4, orange: 60.7, red: 63.1 °C). **(C)** Van’t Hoff relationship between equilibrium constant and temperature for helix 2:CGCAUCCU/AGGAUGCG folding in background monovalent metal ions (240 mM NaCl 140 mM KCl), Eco80, NTPCM, and WMCM. All conditions contain 2 mM free Mg2+. Points and error bars represent association constants and standard errors propagated from the fit (using MeltR). Lines represent the fits to the Van’t Hoff equation that MeltR uses to calculate folding energies. A shift in the Van’t Hoff relationship, down and to the right of the plot area, indicates that Eco80 destabilizes the Helix. **(D)** Helix folding energies in monovalent ions, Eco80, NTPCM, and WMCM were determined using fluorescence binding isotherms that are fit with the MeltR program. All conditions contained 2 mM free Mg2+ as per table 2. The Gibbs free energy at 37 °C, ΔG°37, in Eco80, NTPCM, and WMCM compared to the ΔG37°C in background monovalent metal ions (240 mM NaCl 140 mM KCl), for five RNA helices. All conditions contain 2 mM free Mg2+. Errors were propagated assuming 1.5% uncertainty in the Gibb’s free energy at 37 °C (see methods for error analysis).

Table 3. Stability of RNA helices in *E. coli* metabolite mixtures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sequencea | AU content (%) | Conditionb | ΔG  (kcal/mol)c | ΔΔG  (kcal/mol)c |
| 1: CGGAUGGC/  GCCAUCCG | 25% | 2 mM free | -15.60 (0.23) | -- |
| Eco80 | -15.00 (0.23) | +0.60 (0.32) |
| NTPCM | -15.28 (0.23) | +0.32 (0.33) |
| WMCM | -15.20 (0.23) | +0.40 (0.33) |
| 2: CGCAUCCU/  AGGAUGCG | 38% | 2 mM free | -13.82 (0.21) | -- |
| Eco80 | -12.70 (0.19) | +1.12 (0.28) |
| NTPCM | -13.41 (0.20) | +0.41 (0.29) |
| WMCM | -14.22 (0.21) | -0.40 (0.30) |
| 3: CGUAUGUA/  UACAUACG | 63% | 2 mM free | -10.85 (0.16) | -- |
| Eco80 | -10.41 (0.16) | +0.44 (0.23) |
| NTPCM | -10.30 (0.15) | +0.55 (0.22) |
| WMCM | -10.85 (0.16) | 0.00 (0.23) |
| 4: CCAUAUCA/ UGAUAUGG | 63% | 2 mM free | -12.02 (0.18) | -- |
| Eco80 | -11.38 (0.17) | +0.64 (0.25) |
| NTPCM | -11.50 (0.17) | +0.52 (0.25) |
| WMCM | -11.90 (0.18) | +0.12 (0.25) |
| 5:CCAUAUUA/ UAAUAUGG | 75% | 2 mM free | -10.76 (0.16) | -- |
| Eco80 | -10.15 (0.15) | +0.61 (0.22) |
| NTPCM | -10.16 (0.15) | +0.60 (0.22) |
| WMCM | -9.94 (0.15) | +0.82 (0.22) |

aThe first sequence was 5’-FAM labeled and the second sequence was 3’-BHQ1 labeled. bAll solutions contain 2 mM Free Mg2+ 240 Na+ 140 mM K+.cExtra significant digits were included to avoid propagating rounding errors.

Diagram

Description automatically generated

**Figure 3** *E. coli* metabolite and Mg2+ mixtures stabilize the chemical structure of RNA. **(A)** In-line RNA degradation mechanism facilitated by Mg2+. **(B)** Secondary structure of the guanine riboswitch aptamer with tertiary contacts represented by colored lines. **(C)** Degradation rate, by the increase in counts as a function of time at each residue in different solution conditions as a function of location in the RNA. Degradation fragments greater than 63 could not be resolved on the gel and degradation fragments shorter than 29 were smeared out because of the high concentrations of negatively charged ions in the samples. **(D-F)** Estimated increase in counts as a function of time, in different conditions grouped by the structure each nucleotide, based on analysis of crystal structures and covariation models, into single stranded (SS), meaning that the base was not participating in hydrogen bonding interactions with other residues, non-canonical (NC), meaning that the base was forming non-canonical hydrogen bonding interactions with other residues in the tertiary structure, and Watson-Crick (WC), meaning that the base was in a helix composed mostly of Watson-Crick base pairs

Diagram, schematic

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**Figure 4** *E. coli* metabolite and Mg2+ mixtures support CPEB3 ribozyme catalysis. **(A)** Secondary structure of the un-cleaved CPEB3 ribozyme with the 5’-cleavage site annotated. **(B)** Fraction of cleaved CPEB3 as a function of time fit to a single exponential. Four technical replicates are displayed. 2 mM and 25 mM free refers to the Mg2+ concentration. All conditions contain a background of 240 mM Na+ and 140 mM K+. Enough total Mg2+ was added to Eco80, NTPCM, and WMCM to maintain a 2 mM free Mg2+ concentration. **(C)** First order cleavage-reaction rate constant (k) for the CPEB3 ribozyme in different conditions. krel is the relative rate constant in comparison to the 2 mM free Mg2+ condition. **(D)** Composition of artificial cytoplasms composed of 80% of yeast and mammalian iMBK metabolites, termed Yeast80 and Mammal80 respectively, compared to the composition of Eco80. Each box represents one abundant metabolite. NTPCM represents nucleotide metabolites and WMCM represents metabolites that are expected to weakly chelate Mg2+ with *KD*s greater than 2 mM.

Diagram

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**Diagram, map

Description automatically generatedFigure 5** Models describing the destabilization of RNA helices and stabilization of RNA chemical structure by Eco80. **(A)** Semi-quantitative molecular representation of an RNA helix in Eco80 created with PyMol and WAXSiS. First, the average number of molecules (colored sphere models) in Eco80 that would occupy a sphere with a 50 Å radius was placed randomly around an 8 nucleotide A form RNA helix (blue cartoon, PDB 1SDR). Mg2+ ions are represented with teal spheres. Solvent (red wires) and K+ (blue spheres) where modeled using WAXSiS.53 **(B)** Mechanism for destabilization of helices by metabolites and stabilization of helices by Mg2+. **(C)** Net effect of the metabolite-chelated Mg2+ on the thermodynamic landscape of helix formation combines metabolite interactions favoring the unfolded state of a RNA helix and Mg2+ interactions favoring the helical state. **(D-E)** In-line degradation of the RNA backbone mediated by Mg2+ hydroxide species.

(1) Cech, T. R.; Steitz, J. A. The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. Cell 2014, 157 (1), 77–94. https://doi.org/10.1016/j.cell.2014.03.008.

(2) Ritchey, L. E.; Su, Z.; Tang, Y.; Tack, D. C.; Assmann, S. M.; Bevilacqua, P. C. Structure-Seq2: Sensitive and Accurate Genome-Wide Profiling of RNA Structure in Vivo. Nucleic Acids Res. 2017, 45 (14), e135–e135. https://doi.org/10.1093/nar/gkx533.

(3) Zubradt, M.; Gupta, P.; Persad, S.; Lambowitz, A. M.; Weissman, J. S.; Rouskin, S. DMS-MaPseq for Genome-Wide or Targeted RNA Structure Probing in Vivo. Nat. Methods 2017, 14 (1), 75–82. https://doi.org/10.1038/nmeth.4057.

(4) Lan, T. C. T.; Allan, M. F.; Malsick, L. E.; Woo, J. Z.; Zhu, C.; Zhang, F.; Khandwala, S.; Nyeo, S. S. Y.; Sun, Y.; Guo, J. U.; Bathe, M.; Näär, A.; Griffiths, A.; Rouskin, S. Secondary Structural Ensembles of the SARS-CoV-2 RNA Genome in Infected Cells. Nat. Commun. 2022, 13 (1), 1128. https://doi.org/10.1038/s41467-022-28603-2.

(5) Leamy, K. A.; Assmann, S. M.; Mathews, D. H.; Bevilacqua, P. C. Bridging the Gap between in Vitro and in Vivo RNA Folding. Q. Rev. Biophys. 2016, 49. https://doi.org/10.1017/S003358351600007X.

(6) Nakano, S.; Karimata, H. T.; Kitagawa, Y.; Sugimoto, N. Facilitation of RNA Enzyme Activity in the Molecular Crowding Media of Cosolutes. J. Am. Chem. Soc. 2009, 131 (46), 16881–16888. https://doi.org/10.1021/ja9066628.

(7) Kilburn, D.; Roh, J. H.; Guo, L.; Briber, R. M.; Woodson, S. A. Molecular Crowding Stabilizes Folded RNA Structure by the Excluded Volume Effect. J. Am. Chem. Soc. 2010, 132 (25), 8690–8696. https://doi.org/10.1021/ja101500g.

(8) Kilburn, D.; Roh, J. H.; Behrouzi, R.; Briber, R. M.; Woodson, S. A. Crowders Perturb the Entropy of RNA Energy Landscapes to Favor Folding. J. Am. Chem. Soc. 2013, 135 (27), 10055–10063. https://doi.org/10.1021/ja4030098.

(9) Lee, H.-T.; Kilburn, D.; Behrouzi, R.; Briber, R. M.; Woodson, S. A. Molecular Crowding Overcomes the Destabilizing Effects of Mutations in a Bacterial Ribozyme. Nucleic Acids Res. 2015, 43 (2), 1170–1176. https://doi.org/10.1093/nar/gku1335.

(10) Leamy, K. A.; Yennawar, N. H.; Bevilacqua, P. C. Cooperative RNA Folding under Cellular Conditions Arises From Both Tertiary Structure Stabilization and Secondary Structure Destabilization. Biochemistry 2017, 56 (27), 3422–3433. https://doi.org/10.1021/acs.biochem.7b00325.

(11) Nakano, S.; Karimata, H.; Ohmichi, T.; Kawakami, J.; Sugimoto, N. The Effect of Molecular Crowding with Nucleotide Length and Cosolute Structure on DNA Duplex Stability. J. Am. Chem. Soc. 2004, 126 (44), 14330–14331. https://doi.org/10.1021/ja0463029.

(12) Ghosh, S.; Takahashi, S.; Ohyama, T.; Endoh, T.; Tateishi-Karimata, H.; Sugimoto, N. Nearest-Neighbor Parameters for Predicting DNA Duplex Stability in Diverse Molecular Crowding Conditions. Proc. Natl. Acad. Sci. 2020, 117 (25), 14194–14201. https://doi.org/10.1073/pnas.1920886117.

(13) Adams, M. S.; Znosko, B. M. Thermodynamic Characterization and Nearest Neighbor Parameters for RNA Duplexes under Molecular Crowding Conditions. Nucleic Acids Res. 2019, 47 (7), 3658–3666. https://doi.org/10.1093/nar/gkz019.

(14) Tyrrell, J.; Weeks, K. M.; Pielak, G. J. Challenge of Mimicking the Influences of the Cellular Environment on RNA Structure by PEG-Induced Macromolecular Crowding. Biochemistry 2015, 54 (42), 6447–6453. https://doi.org/10.1021/acs.biochem.5b00767.

(15) Stadmiller, S. S.; Pielak, G. J. Protein-Complex Stability in Cells and in Vitro under Crowded Conditions. Curr. Opin. Struct. Biol. 2021, 66, 183–192. https://doi.org/10.1016/j.sbi.2020.10.024.

(16) You, X.; Nguyen, A. W.; Jabaiah, A.; Sheff, M. A.; Thorn, K. S.; Daugherty, P. S. Intracellular Protein Interaction Mapping with FRET Hybrids. Proc. Natl. Acad. Sci. 2006, 103 (49), 18458–18463. https://doi.org/10.1073/pnas.0605422103.

(17) Phillip, Y.; Kiss, V.; Schreiber, G. Protein-Binding Dynamics Imaged in a Living Cell. Proc. Natl. Acad. Sci. 2012, 109 (5), 1461–1466. https://doi.org/10.1073/pnas.1112171109.

(18) Sukenik, S.; Ren, P.; Gruebele, M. Weak Protein–Protein Interactions in Live Cells Are Quantified by Cell-Volume Modulation. Proc. Natl. Acad. Sci. 2017, 114 (26), 6776–6781. https://doi.org/10.1073/pnas.1700818114.

(19) Hull, C. M.; Bevilacqua, P. C. Discriminating Self and Non-Self by RNA: Roles for RNA Structure, Misfolding, and Modification in Regulating the Innate Immune Sensor PKR. Acc. Chem. Res. 2016, 49 (6), 1242–1249. https://doi.org/10.1021/acs.accounts.6b00151.

(20) Uehata, T.; Takeuchi, O. RNA Recognition and Immunity—Innate Immune Sensing and Its Posttranscriptional Regulation Mechanisms. Cells 2020, 9 (7), 1701. https://doi.org/10.3390/cells9071701.

(21) Yamagami, R.; Sieg, J. P.; Bevilacqua, P. C. Functional Roles of Chelated Magnesium Ions in RNA Folding and Function. Biochemistry 2021, 60 (31), 2374–2386. https://doi.org/10.1021/acs.biochem.1c00012.

(22) Yamagami, R.; Bingaman, J. L.; Frankel, E. A.; Bevilacqua, P. C. Cellular Conditions of Weakly Chelated Magnesium Ions Strongly Promote RNA Stability and Catalysis. Nat. Commun. 2018, 9 (1), 2149. https://doi.org/10.1038/s41467-018-04415-1.

(23) Yamagami, R.; Huang, R.; Bevilacqua, P. C. Cellular Concentrations of Nucleotide Diphosphate-Chelated Magnesium Ions Accelerate Catalysis by RNA and DNA Enzymes. Biochemistry 2019, 58 (38), 3971–3979. https://doi.org/10.1021/acs.biochem.9b00578.

(24) Leamy, K. A.; Yamagami, R.; Yennawar, N. H.; Bevilacqua, P. C. Single-Nucleotide Control of TRNA Folding Cooperativity under near-Cellular Conditions. Proc. Natl. Acad. Sci. U. S. A. 2019, 116 (46), 23075–23082. https://doi.org/10.1073/pnas.1913418116.

(25) Bennett, B. D.; Kimball, E. H.; Gao, M.; Osterhout, R.; Van Dien, S. J.; Rabinowitz, J. D. Absolute Metabolite Concentrations and Implied Enzyme Active Site Occupancy in Escherichia Coli. Nat. Chem. Biol. 2009, 5 (8), 593–599. https://doi.org/10.1038/nchembio.186.

(26) Martell, A. E.; Smith, R. M. Critical Stability Constants; NewYork ; London : Plenum Press, 1974; Vol. V1.

(27) Martell, A. E.; Smith, R. M. Critical Stability Constants; New York, Plenum Press, 1974; Vol. V2.

(28) Martell, A. E.; Smith, R. M. Critical Stability Constants; New York, Plenum Press, 1974; Vol. V3.

(29) Martell, A. E.; Smith, R. M. Critical Stability Constants.; New York ; London : Plenum, 1982; Vol. V5.

(30) Smith, R. M. (Robert M.; Martell, A. E. Critical Stability Constants; New York ; London : Plenum, 1989; Vol. V6.

(31) Berthon, G. Critical evaluation of the stability constants of metal complexes of amino acids with polar side chains (Technical Report). Pure Appl. Chem. 1995, 67 (7), 1117–1240. https://doi.org/10.1351/pac199567071117.

(32) Martell, A. E.; Smith, R. M. NIST46 Critically Selected Stability Constants of Metal Complexes. NIST. https://www.nist.gov/srd/nist46 (accessed 2021-05-25).

(33) Grilley, D.; Soto, A. M.; Draper, D. E. Chapter 3 Direct Quantitation of Mg2+‐RNA Interactions by Use of a Fluorescent Dye. In Methods in Enzymology; Biothermodynamics, Part A; Academic Press, 2009; Vol. 455, pp 71–94. https://doi.org/10.1016/S0076-6879(08)04203-1.

(34) Puglisi, J. D.; Tinoco, I. [22] Absorbance Melting Curves of RNA. In Methods in Enzymology; RNA Processing Part A: General Methods; Academic Press, 1989; Vol. 180, pp 304–325. https://doi.org/10.1016/0076-6879(89)80108-9.

(35) Xia, T.; SantaLucia, J.; Burkard, M. E.; Kierzek, R.; Schroeder, S. J.; Jiao, X.; Cox, C.; Turner, D. H. Thermodynamic Parameters for an Expanded Nearest-Neighbor Model for Formation of RNA Duplexes with Watson−Crick Base Pairs †. Biochemistry 1998, 37 (42), 14719–14735. https://doi.org/10.1021/bi9809425.

(36) Liu, B.; Shankar, N.; Turner, D. H. Fluorescence Competition Assay Measurements of Free Energy Changes for RNA Pseudoknots. Biochemistry 2010, 49 (3), 623–634. https://doi.org/10.1021/bi901541j.

(37) Liu, B.; Diamond, J. M.; Mathews, D. H.; Turner, D. H. Fluorescence Competition and Optical Melting Measurements of RNA Three-Way Multibranch Loops Provide a Revised Model for Thermodynamic Parameters. Biochemistry 2011, 50 (5), 640–653. https://doi.org/10.1021/bi101470n.

(38) You, Y.; Tataurov, A. V.; Owczarzy, R. Measuring Thermodynamic Details of DNA Hybridization Using Fluorescence. Biopolymers 2011, 95 (7), 472–486. https://doi.org/10.1002/bip.21615.

(39) Adamala, K.; Szostak, J. W. Non-Enzymatic Template-Directed RNA Synthesis inside Model Protocells. Science 2013, 342 (6162), 1098–1100. https://doi.org/10.1126/science.1241888.

(40) Soukup, G. A.; Breaker, R. R. Relationship between Internucleotide Linkage Geometry and the Stability of RNA. RNA 1999, 5 (10), 1308–1325.

(41) Mandal, M.; Boese, B.; Barrick, J. E.; Winkler, W. C.; Breaker, R. R. Riboswitches Control Fundamental Biochemical Pathways in Bacillus Subtilis and Other Bacteria. Cell 2003, 113 (5), 577–586. https://doi.org/10.1016/S0092-8674(03)00391-X.

(42) Batey, R. T.; Gilbert, S. D.; Montange, R. K. Structure of a Natural Guanine-Responsive Riboswitch Complexed with the Metabolite Hypoxanthine. Nature 2004, 432 (7015), 411. https://doi.org/10.1038/nature03037.

(43) Gilbert, S. D.; Love, C. E.; Edwards, A. L.; Batey, R. T. Mutational Analysis of the Purine Riboswitch Aptamer Domain †. Biochemistry 2007, 46 (46), 13297–13309. https://doi.org/10.1021/bi700410g.

(44) Poudyal, R. R.; Sieg, J. P.; Portz, B.; Keating, C. D.; Bevilacqua, P. C. RNA Sequence and Structure Control Assembly and Function of RNA Condensates. RNA 2021, 27 (12), 1589–1601. https://doi.org/10.1261/rna.078875.121.

(45) Park, J. O.; Rubin, S. A.; Xu, Y.-F.; Amador-Noguez, D.; Fan, J.; Shlomi, T.; Rabinowitz, J. D. Metabolite Concentrations, Fluxes and Free Energies Imply Efficient Enzyme Usage. Nat. Chem. Biol. 2016, 12 (7), 482–489. https://doi.org/10.1038/nchembio.2077.

(46) Klosin, A.; Oltsch, F.; Harmon, T.; Honigmann, A.; Jülicher, F.; Hyman, A. A.; Zechner, C. Phase Separation Provides a Mechanism to Reduce Noise in Cells. Science 2020, 367 (6476), 464–468. https://doi.org/10.1126/science.aav6691.

(47) Riback, J. A.; Zhu, L.; Ferrolino, M. C.; Tolbert, M.; Mitrea, D. M.; Sanders, D. W.; Wei, M.-T.; Kriwacki, R. W.; Brangwynne, C. P. Composition-Dependent Thermodynamics of Intracellular Phase Separation. Nature 2020, 581 (7807), 209–214. https://doi.org/10.1038/s41586-020-2256-2.

(48) Lambert, D.; Draper, D. E. Effects of Osmolytes on RNA Secondary and Tertiary Structure Stabilities and RNA-Mg2+ Interactions. J. Mol. Biol. 2007, 370 (5), 993–1005. https://doi.org/10.1016/j.jmb.2007.03.080.

(49) Pegram, L. M.; Wendorff, T.; Erdmann, R.; Shkel, I.; Bellissimo, D.; Felitsky, D. J.; Record, M. T. Why Hofmeister Effects of Many Salts Favor Protein Folding but Not DNA Helix Formation. Proc. Natl. Acad. Sci. 2010, 107 (17), 7716–7721. https://doi.org/10.1073/pnas.0913376107.

(50) Lambert, D.; Draper, D. E. Denaturation of RNA Secondary and Tertiary Structure by Urea: Simple Unfolded State Models and Free Energy Parameters Account for Measured m-Values. Biochemistry 2012, 51 (44), 9014–9026. https://doi.org/10.1021/bi301103j.

(51) Cheng, X.; Shkel, I. A.; Molzahn, C.; Lambert, D.; Karim, R.; Record, M. T. Quantifying Interactions of Nucleobase Atoms with Model Compounds for the Peptide Backbone and Glutamine and Asparagine Side Chains in Water. Biochemistry 2018, 57 (15), 2227–2237. https://doi.org/10.1021/acs.biochem.8b00087.

(52) Williams, A. M.; Dickson, T.; Lagoa-Miguel, C.; Bevilacqua, P. C. Biological Solution Conditions and Flanking Sequence Modulate LLPS of RNA G-Quadruplex Structures. RNA 2022, rna.079196.122. https://doi.org/10.1261/rna.079196.122.

(53) Knight, C. J.; Hub, J. S. WAXSiS: A Web Server for the Calculation of SAXS/WAXS Curves Based on Explicit-Solvent Molecular Dynamics. Nucleic Acids Res. 2015, 43 (W1), W225–W230. https://doi.org/10.1093/nar/gkv309.