The Metabolome Weakens RNA Thermodynamic Stability and  
 Strengthens RNA Chemical Stability

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Description automatically generatedWe examined the complex network of interactions amongst RNA, the metabolome, and divalent Mg2+ under 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 the development of an artificial cytoplasm that mimics *in vivo E. coli* conditions, which we term “Eco80”. We empirically determined that the mixture of *E. coli* metabolites in Eco80 approximated 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 found that Eco80conditions lead to opposing effects on the thermodynamic and chemical stability of RNA. In particular, the thermodynamic stability of RNA helices was weakened by 0.69±0.12 kcal/mol while the chemical stability was enhanced ~2-fold, which can be understood using the speciation of Mg2+ between weak and strong Mg2+-metabolite complexes in Eco80. Overall, the use of Eco80 reflects RNA function *in vivo* and enhances the biological relevance of mechanistic studies of RNA.

**Introduction**

RNA serves as the conduit of genetic information in the Central Dogma of Molecular Biology and performs numerous functions in cells owing to its capacity to form complex, diverse, and functional structures.1 The development of genome wide structure-probing techniques *in vivo* has provided insight into RNA structure and function in cells.2–5 However, most experimental techniques that provide 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 1,000 mM monovalent metal ions and 0.5 to 50 mM free divalent magnesium ions (Mg2+) with a dilute buffer.6 *In vitro* studies of RNA in conditions that mimic a cell, so called *in vivo*-like conditions, provide a link between experiments that probe RNA structure *in vivo* and experiments that provide mechanistically-tractable biologically-relevant insight *in* *vitro*.6

Many studies have investigated the effects of individual components of the cellular environment on nucleic acid structure, including small molecules and non-biological crowders. Studies that used small molecules that are similar to metabolites indicated that these species interact strongly with the unfolded state of nucleic acids and destabilize secondary structure.1–5 Studies that simulate cellular macromolecules revealed stabilized RNA tertiary structures, increased folding cooperativity, and improved RNA function in crowded enviroments.7–11 Thermodynamic characterization of RNA helix formation in crowding conditions indicated that crowders generally destabilize helices.12–14 In summary, using simple models to simulate the cellular environment has provided valuable insight into how the cell affects nucleic acids and motivated investigation of more complex and realistic artificial cytoplasms.15

A number of studies have provided mechanistic insight into proteins in complex environments,16 ranging from cell lysates to live cells.17–19 However, researchers sacrifice the control over the environment that is provided by a simple system. Mechanistic studies of RNA in cells or lysates have two additional problems. The first is the propensity of cells to degrade foreign RNA.20,21 The second is the lack of control over Mg2+ speciation between free and chelated Mg2+.

Control over Mg2+ speciation is crucial for mechanistic studies of RNA because of the sensitivity of RNA folding to the concentration of Mg2+ in the solution, as demonstrated by thousands of studies and summarized.22 Furthermore, recent studies have demonstrated the importance of metabolite-chelated Mg2+ complexes to RNA function.23–25 These studies considered 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, in these studies, Mg2+ speciation was approximated assuming single-site binding, meaning that one metabolite interacts with one Mg2+ ion, and binding constants were extrapolated from published sources, reported at disparate ionic compositions and pHs.22

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 biologically relevant pH and ionic strength, and lastly determine the total Mg2+ concentration in the final mixture of metabolites. This *aufbau* approach allows us to study the effects of the metabolite and metal ion species that comprise a major portion of the interactions that RNA experiences in *E. coli* cells.

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

aUncertainty is in parentheses and is 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 ITC 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 the free Mg2+ concentration in *E. coli* of 2 mM (top rows of table) are considered strong Mg2+ chelators and denoted “Strong” and *KD*s greater than 2 mM (bottom rows of table) are considered weak Mg2+ chelators and denoted “Weak”. Sub-artificial cytoplasms comprising Eco80, nucleotide triphosphate-chelated Mg2+ (NTPCM) and weak metabolite-chelated Mg2+ (WMCM) are noted.

**Results**

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

*E. coli* cells contain hundreds of different metabolites (~240 mM total),26 which is too many to test systematically. However, the 15 most abundant metabolites in *E. coli*, an experimentally-manageable number, comprise a full 80% (195 mM) of the 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 were zwitterions or negatively charged near physiological pH 7, the latter requiring electrostatic neutralization with monovalent metal ions. Metabolite salts and free acids were prepared to a final 2x concentration, and the amount of Na+ and K+ added with each metabolite was recorded. Next, the pH of the 2x stock was adjusted to pH 7.0 using NaOH, and the amount of Na+ was recorded. Lastly, NaCl and KCl were added to final concentrations of 480 mM Na+ and 280 mM K+, twice the physiological value of 240 mM Na+ and 140 mM K+.6 The 2x-concentrated artificial cytoplasm was then diluted into other reagents to give the final 1x concentration required for experiments.

Next, we considered how metabolites affect the speciation of Mg2+ between free and chelated forms. All 15 Eco80 metabolites have functional groups, phosphates and carboxylates, that drive chelating interactions with Mg2+ ions (Table 1).22 We sought to quantify Mg2+ chelation by the metabolites inEco80at the physiological background, since Mg2+ binding affinity is dependent on environmental factors such as pH, ionic strength and identify, and temperature.27–33

We determined apparent disassociation constants (*KD*) for Eco80 metabolites in a background of 240 mM NaCl, 140 mM KCl, and 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 divalent metal ion-binding dye 8-hydroxy-5-quinolinesulfonic (HQS) acid,34 was used to estimate the *KD*s 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+ was titrated into HQS solutions in the absence and presence of Mg2+ chelators. First, emission of HQS as a function of the total Mg2+ in the absence of chelators was independently performed in each panel and fit to a binding model for the binding of Mg2+ to HQS (SI Figure 2 top black data and fit). The free Mg2+ concentration, which is equal to the total Mg2+ concentration in this case, 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 the free Mg2+ concentration at any total concentration of Mg2+ (SI Figure 2, bottom). Note that free and total Mg2+ concentrations are the same, y=x, in the absence of chelators, and that the data were right-shifted in the presence of chelators. The affinity of Mg2+ binding by metabolite was thus obtained by fitting the free Mg2+ concentration as a function of the total Mg2+ concentration.

The binding affinity between Eco80 metabolites and Mg2+ ranged from strong to negligible (Table 1). The four most abundant nucleotide triphosphates, ATP, UTP, GTP, and dTTP, were classified as strong Mg2+ binders, with *KD* values ranging from 0.160±0.003 mM to 0.28±0.01 mM, less than the approximate free Mg2+ concentration in *E. coli* of 2 mM (Table 1). Conversely, 8 metabolites--L-glutamic acid, fructose 1,6-bisphosphate, 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 other 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 the weak/non Mg2+ chelators, respectively (Table 1).

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Figure 1 Analysis of Mg2+ speciation in *E. coli* metabolitemixtures. (A) *E. coli* metabolome molar composition. ‘Eco80’ contains the 15 most abundant metabolites, which comprise 80% of the *E. coli* metabolome. ‘NTPCM’ contains the four strong Mg2+ chelating NTPs, and ‘WMCM’ contains 11 other weak Mg2+-binding metabolites. (B-D) Effect of Mg2+ on HQS emission without and with mixtures of metabolites that chelate Mg2+. Grey lines represent fits to determine the binding constant between Mg2+ and HQS. (E-G) Relationship between free Mg2+ concentration and the total Mg2+ concentration with mixtures of metabolites that chelate Mg2+. Hex bins represent a range of total and free Mg2+ concentrations simulated from artificial cytoplasm assuming single-site binding (colors correspond to density of simulated values in a hex bin, with yellow being most dense and purple being least dense). Triangle data points (black) are free Mg2+ concentrations calculated using HQS emission. Black lines were generated using polynomial regression. The red shaded region is the biological free Mg2+ range of 0.5 to 3 mM. The red line is the approximate free Mg2+ concentration in *E. coli* of 2 mM. Downward red arrows represent the total Mg2+required to maintain 2 mM free Mg2+.

We used two methods to estimate how Eco80 metabolites affect the speciation of Mg2+ between free and chelated. Our first method was the HQS assay that we used to estimate binding constants for metabolites, based on calculating the free Mg2+ concentration in the presence of metabolites using HQS fluorescence emission (Figure 1B-D, SI Table 4). The advantage of this method was that it directly determines the concentration of free Mg2+; it does not, however, report on speciation of Mg2+ to different metabolites. Our second method used a statistical model that accounts for experimental uncertainties in metabolite concentrations and *KD* values and estimates Mg2+ speciation assuming single-site binding (meaning that one metabolite associated one Mg2+ ion). The advantage of this method was that it approximated Mg2+ speciation to different metabolites; it did not, however, directly determine free Mg2+ concentrations. This statistical model is described in detail in the Supporting 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 a series of virtual artificial cytoplasm with different errors. [Mg]T is the total Mg2+ concentration, [Mg] is the free Mg2+ concentration, N is the total number of metabolites in a mixture, “*i"* is an integer representing each metabolite in a mixture, [Li]T is the total concentration of the ith metabolite in a mixture, and *KD* is the dissociation constant of the ith metabolite.

Then, Equation 1 was solved numerically 1000 different times to determine a range of free Mg2+ concentrations produced at a given total Mg2+ concentration in a virtual artificial cytoplasm.

On the basis of agreement between the HQS data and the statistical simulation, methods 1 and 2, respectively, the two methods supported a model in which Mg2+ speciated in artificial cytoplasms largely according to single-site binding within the biological free Mg2+ concentration range of 0.5 to 3 mM Mg2+. However, at higher free Mg2+ concentrations, Mg2+ did not speciate according to a single-site model (Figure 1 E-G, black data points and hex bins deviate from each other).

In Eco80, the statistical model indicated 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 over this range with HQS emission were consistent with this single-site behavior (Figure 1E). At higher free Mg2+ concentrations, Eco80 was expected to lose its free Mg2+-buffering capacity as chelators become saturated, and the free Mg2+ should have increased sharply with the total Mg2+ (model in Figure 1E, hex bins). However, the free Mg2+ concentration measured with HQS did not increase as fast as the statistical model predicted above 3 mM free Mg2+ (Figure 1E, compare black data points and hex bins). 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 increased from 3 mM to ~10 mM (Figure 1E, data points). One possibility is that multivalent interactions, where several Mg2+-saturated metabolites interact with additional Mg2+ molecules, dominate the equilibrium. Such non-single-site behavior above 3 mM free Mg2+ was also observed in the NTPCM and WMCM artificial cytoplasms (Figure 1 F & G), and was observed previously.22

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 cytoplasms. The relationship between the free Mg2+ calculated from HQS emission and the total Mg2+ concentration in each artificial cytoplasm 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 6.4 mM total Mg2+ to produce 2 mM free Mg2+ in Eco80, NTPCM, and WMCM, respectively (Table 2).

Table 2. 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 | 4.4 | 2.0 |

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Figure 2 *E. coli* metabolite-Mg2+ mixtures destabilized RNA helices. (A) Layout of a fluorescence-detected binding isotherm assay in a real-time PCR machine. FAM emission is normalized to a passive ROX reference dye. (B) Fluorescence-detected binding isotherms fit to determine equilibrium constants with MeltR. Data points represent raw fluroescence 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 the helix association equilibrium constant and temperature for helix 2:CGCAUCCU/AGGAUGCG folding in background, Eco80, NTPCM, and WMCM. All conditions contain 2 mM free Mg2+, 240 mM Na+, and 140 mM K+. Points and error bars represent association constants and standard errors propagated from the fit (using MeltR). Lines represent fits to the Van’t Hoff equation that MeltR used to calculate folding energies. (D) The Gibbs free energy at 37 °C (ΔG°37) in Eco80, NTPCM, or WMCM compared to the ΔG°37 in background for five RNA helices. Errors were propagated assuming 1.5% uncertainty in the ΔG°37 (see methods for error analysis).

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

We sought to understand how Eco80 affects the thermodynamic stability of RNA. Stability of RNA helices is traditionally measured with UV absorbance-detected melting curves, typically monitored at 260 or 280 nm.35,36 However, absorbance melting curves could not measure helix stability in Eco80 because of the high absorptivity of the nucleotide metabolites. Thus, we pursued a fluorescence-detected binding isotherm assay.

Helix stability was monitored using 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 RNA-BHQ1. We used a binding isotherm method, wherein decreasing concentrations of RNA-BHQ1 are titrated into a constant concentration of FAM-RNA (SI Figure 3), resulting in a binding isotherm (Figure 2B). We favored binding isotherms over fluorescence-detected melts because of the very strong dependence of FAM emission on temperature.37–39 Emission of FAM was monitored at different temperatures, resulting in an isotherm every 0.5 °C from 20 to 80 °C. Figure 2B shows a subset of these 121 isotherms.

Raw fluorescence data were fit with MeltR, a program created by the authors, to determine folding energies. MeltR is a package of functions in the R programming language that allowed facile conversion of raw data to folding energies (see SI methods for details). MeltR calculated 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.

*Eco80 thermodynamically destabilizes RNA helices*

We used fluorescence-detected binding isotherms to determine helix folding energies in a background control of 240 mM NaCl and 140 mM KCl, and either Eco80, NTPCM, and WMCM for a set of five representative eight base-pair RNA helices; all solutions contained 2 mM free Mg2+(Table 2). This helix set was designed to contain one or more representatives of each of the 10 Watson-Crick nearest neighbor parameters and vary in AU content from 25% to 75% (Table 3). Both of the aforementioned methods to determine folding free energies in MeltR agreed (SI Table 5); the results from the Van’t Hoff plot are reported in Table 3, which is ranked according to the AU content of the duplex. Errors in the main text are reported as 1.5% in terms of the ΔG°37 and a detailed error analysis is available in the SI methods.

All five representative helices were significantly destabilized in Eco80 relative to the background condition, meaning the ΔΔG°37 between the background condition and Eco80 was larger than its propagated uncertainty (Table 3, Figure 2D). Destabilization ΔΔG°37 values ranged from +0.44±0.23 to +1.17±0.28 kcal/mol, with an average value of +0.69±0.12 kcal/mol (Table 3). We did not observe a clear relationship between AU content and destabilization (SI Figure 4). Thus, Eco80 destabilized RNA helices but the underlying sequence dependence was not apparent.

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sequencea | AU content (%) | Conditionb | ΔG°37 (kcal/mol)c | ΔΔG°37 (kcal/mol)c |
| 1:  5’-CGGAUGGC-3’  3’-GCCUACCG-5’ | 25% | Background | -15.60 (0.23) | -- |
| Eco80 | -14.99 (0.22) | +0.61 (0.32) |
| NTPCM | -15.28 (0.23) | +0.32 (0.33) |
| WMCM | -15.23 (0.23) | +0.37 (0.33) |
| 2:  5’-CGCAUCCU-3’  3’-GCGUAGGA-5’ | 38% | Background | -13.84 (0.21) | -- |
| Eco80 | -12.70 (0.19) | +1.17 (0.28) |
| NTPCM | -13.42 (0.20) | +0.42 (0.29) |
| WMCM | -14.22 (0.21) | -0.39 (0.30) |
| 3:  5’-CGUAUGUA-3’  3’-GCAUACAU-5’ | 63% | Background | -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:  5’-CCAUAUCA-3’  3’-GGUAUAGU-5’ | 63% | Background | -12.03 (0.18) | -- |
| Eco80 | -11.38 (0.17) | +0.64 (0.25) |
| NTPCM | -11.50 (0.17) | +0.52 (0.25) |
| WMCM | -11.53 (0.17) | +0.50 (0.25) |
| 5:  5’-CCAUAUUA-3’  3’-GGUAUAAU-5’ | 75% | Background | -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) | +0.80 (0.22) |

aThe first sequence in each set was 5´-FAM labeled while 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.

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), with ΔΔG°37 values ranging from +0.32±0.33 to 0.64±0.23 kcal/mol, with an average value of +0.48±0.12 kcal/mol (Table 3). The destabilizing effect of Eco80 appeared 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 = 0.9, SI Figure 4). This could be because the NTPCM is comprised solely of NTPs and these can base pair most readily with A and U (see Discussion).

In contrast, WMCM, which is comprised 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). The ΔΔG°37 values ranged from ‑0.39±0.30 to +0.80±0.22 kcal/mol, with an average value of +0.26±0.12 kcal/mol, hardly above the noise (Table 3). Similar to Eco80, the sequence dependence of stabilization or destabilization was not clear (SI Figure 4).

Overall, the net effect of Eco80 on RNA helices was 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.

*Eco80 protects RNA from chemical degradation*

Several studies indicated that weak and strong Mg2+ chelating metabolites reduced Mg2+-mediated RNA degradation.23,40 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.41 For ILP, the 2´-hydroxyl is deprotonated by a Mg2+-hydroxide (Mg2+-OH-), and serves as a nucleophile to attack the adjacent phosphate in a 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.41 For this assay, 5´-32P RNAs were incubated at 37 °C up to 90 h to facilitate in-line cleavage, with time points taken regularly. RNA fragments were then fractionated on a denaturing PAGE gel (SI Figure 5), providing single-nucleotide resolution of RNA degradation rates measured by the increase in counts with time for a given band. In-line degradation rates for 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 a 25 mM free Mg2+ condition. All conditions contained 240 mM K+ and 140 mM Na+. 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 mM total Mg2+ concentration used for NTPCM and Eco80, respectively (Table 2).

We first used ILP cleaved versus time data to determine degradation rates for the guanine riboswitch aptamer (Figure 3B) in different artificial cytoplasms. The guanine riboswitch aptamer has been studied extensively, providing structural and mechanistic information.42–44. 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 favor the apo state. Moreover, guanine binding to the aptamer induces structural changes only at nucleotides directly mediating the guanine binding site,42 indicating that information provided by X-ray crystal structures of the ligand bound aptamer is relevant for a structural analysis of our degradation rates.

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 Mg2+, Eco80, NTPCM, and WMCM conditions, with high degradation in the 3’-region of the P2 stem and high reactivity in the L3 region, 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 was similar to ILP data published for another guanine riboswitch at a higher pH and a Mg2+ concentration of 15 mM,45 supporting that the increase in degradation rates in the 25 mM free Mg2+ condition was dependent on the presence of Mg2+-OH- complexes (SI Figure 6).

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**Figure 3** *E. coli* metabolite-Mg2+ mixtures stabilized the chemical structure of RNA. **(A)** ILP degradation mechanism facilitated by free Mg2+(OH-). **(B)** Secondary structure of the guanine riboswitch aptamer with tertiary contacts.43 **(C)** Degradation rate at each residue in different solution conditions. **(D-F)** Degradation rate in different conditions grouped by structure. Groupings were based on analysis of crystal structures. SS: Single-stranded, the base was not participating in hydrogen bonding interactions with other residues. NC: non-canonical, the base was forming non-canonical hydrogen bonding interactions in the tertiary structure. WC: Watson-Crick, the base was in a helix composed mostly of Watson-Crick base pairs.

To further confirm that the guanine aptamer adopts similar structures in all conditions, we collected small angle X-ray scattering (SAXS) data on the apo form of the aptamer. Bell-shaped Kratky plots indicated that the structure of the guanine aptamer is folded between conditions (SI Figure 7A). To better understand how the solution state compares to the crystal structure, we generated a modeled SAXS curve from the crystal structure of the guanine aptamer (PDB 4FE5)43 using WAXSiS to account for scattering by the ordered-solvent layer (SI Figure 7A, black line).46 The predicted 19.7±0.2 Å radius of gyration (Rg) of the model was smaller than the Rg calculated using Guinier analysis of the solution state, 24.5±0.2 Å in 2 mM free Mg2+, 23.9±0.2 Å in Eco80, 24.5±0.4 Å in NTPCM, 26.9±0.5 Å in WMCM, and 27.1±0.2 Å in 2 mM free Mg2+ (SI Table 6). These trends in radius of gyration were reproduced using paired-distance distribution analysis and *ab-inito*-electron-density reconstruction (SI Table 6),47 indicating that the guanine riboswitch aptamer adapts an expanded structure in solution compared to its crystal structure. Likewise, distance distributions in solution are right shifted in comparison to the crystal structure (SI Figure 7B), and the shape of electron-density reconstructions (SI Figure 7C-G) are consistent with the crystal structure of the guanine riboswitch aptamer adopting expanded states in solution.

A more detailed interpretation of the SAXS data between solution conditions was confounded by the high noise in our data, ambiguous determination of the maximum distance between atoms (SI Figure 7B, the distribution does not approach a limit at y=0), and inability to deconvolute scattering due to compaction and due to the composition of the ordered-solvent layer. However, there was consistent support for structural compaction in Eco80, with the radius of gyration changing from 24.5±0.2 to 23.9±0.4 Å, the Dmax changing from 69.4 to 65.4 Å, and the excluded volume changing from 42,787 to 33,212 Å in 2mM free Mg2+ and Eco80, respectively (SI Table 6). This structural compaction in Eco80 was similar to the structural compaction previously observed in crowded conditions.8,11

We sought to better characterize the structural dependence of RNA degradation in different conditions. We therefore extended the study to two other RNAs with well-defined tertiary structures, the CPEB3 ribozyme48,49 and yeast tRNAPhe (SI Figure 8, 9, & 10). 50 We inspected the crystal structures of these two RNAs, plus the original guanine aptamer, and manually classified each residue 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 comprised 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 for nucleotides involved in non-canonical tertiary interactions (NC) in 2 mM free Mg2+, Eco80, NTPCM, and WMCM in comparison to the 25 mM free Mg2+ condition. 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 degradation in artificial cytoplasm for SS and NC bases specifically, even with similar amounts of total Mg2+ in solution as the 25 mM free Mg2+ condition.

We repeated our degradation assay with the self-cleaved CPEB3 ribozyme and yeast tRNAphe, to test if the protection from 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 that degradation rates were partially dependent on the strength of Mg2+ chelation. Likewise, the degradation rates for residues that were predicted to participate in 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 that degradation rates were 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 10D). Single-stranded nucleotides showed reduced degradation rates in the 2 mM free Mg2+, Eco80, and NTPCM conditions in comparison to the 25 mM free Mg2+ condition, and degradation rates recovered in WMCM (Figure 3F). Degradation rates were constant across conditions for nucleotides that form Watson-Crick base pairs and non-canonical contacts, which is different than the increased degradation observed for nucleotides that form non-canonical contacts 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.

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 both artificial cytoplasms have relatively high concentrations of total Mg2+. WMCM showed an intermediate effect between the high degradation rates in the 25 mM free Mg2+ condition and the low degradation rates in 2 mM free Mg2+, Eco80, and NTPCM conditions, indicating that degradation rates were 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 ~1.6-fold by an estimated 2 mM free Mg2+ in solution with 11.3 mM glutamate-chelated Mg2+, in comparison to catalysis in 2 mM free Mg2+ alone.23 Thus, we 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). All conditions contained 240 mM Na+ and 140 mM K+. Briefly, we purified full length CPEB3 ribozyme (Figure 4A), incubated it in artificial cytoplasm, fractionated time points on a denaturing acrylamide gel, and calculated the fraction cleaved from the relative intensity of cleaved and uncleaved RNA bands (SI Figure 11). The fraction cleaved as a function of time was fit to a single-exponential equation to estimate the reaction rate constant (Figure 4B).

In comparison to the 2 mM free Mg2+ control, CPEB3 ribozyme catalysis was modestly reduced in all conditions (Figure 4C). Surprisingly, CPEB3 catalysis was reduced in Eco80 by ~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 ~1/3 in NTPCM in comparison to the 2 mM free Mg2+ control, an slightly stronger inhibitory effect than Eco80. In contrast, CPEB3 catalysis was enhanced ~1.3 fold in WMCM, similar to the enhancement observed for glutamate-chelated Mg2+.23 In summary, Eco80 supports RNA catalysis albeit not in an enhanced fashion in comparison to the 2 mM free Mg2+ condition. CPEB3 reaction rates in Eco80 were between the rates in WMCM and NTPCM. WMCM likely had exposed Mg2+ to help fold the RNA, while NTPCM did not, and moreover may denature the RNA as per Figure 2 (see Discussion).

Diagram

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Figure 4 Eco80 supports CPEB3-ribozyme catalysis. (A) Secondary structure of the uncleaved CPEB3 ribozyme. (B) Fraction cleaved CPEB3 as a function of time fit to a single exponential. Four technical replicates are displayed. The labels ‘2 mM Free’ and ‘25 mM Free’ refer 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) Rate constant (*k*) for the CPEB3 ribozyme in different conditions. *k*rel is the relative rate constant in comparison to the 2 mM free Mg2+ condition. (D) Composition of artificial cytoplasms comprised 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 which were expected to weakly chelate Mg2+ with KDs greater than 2 mM.

WMCM may be more biologically relevant than Eco80 for studying CPEB3 ribozyme activity. We performed an analysis of absolute metabolite concentrations in yeast and mammalian iMBK cells, which have a closer evolutionary relationship than *E. coli* to human cells, where CPEB3 exists in nature (Figure 4D). Absolute metabolite concentrations were compiled from the literature51 and the 11 most abundant metabolites that comprise 80% of the yeast and mammalian metabolome were selected to assess hypothetical Yeast80 and Mammal80 artificial cytoplasms. Estimated metabolite-Mg2+ binding constants22 were used to classify each metabolite in Yeast80 and Mammal80 as strong (NTPCM) or weak Mg2+ chelators. We found that Yeast80 and Mammal80 would be depleted in strongly chelated-Mg2+, with Yeast80 having no strong Mg2+ chelators and Mammal80 having only ~4 mM strong Mg2+ chelators. (Figure 4D). Thus, the 1.3-fold rate enhancement in WMCM is more relevant to CPEB3 function in human cells than the rate decrease in Eco80.

**Discussion**

In this study, we used a bottom-up, *aufbau*, approach to create a complex, yet still manageable artificial cytoplasm, termed Eco80, which encapsulated 80% of the *E. coli* metabolome (Figure 5A). In order to provide mechanistic insight into the effects of Mg2+ speciation on RNA in cells, we also broke down Eco80 into sub-artificial cytoplasms, which contain either metabolites that strongly chelate Mg2+ (i.e. NTPs), or metabolites that weakly chelate Mg2+.

Importantly, we adopted the Mg2+ sensitive dye, HQS,34 to measure Mg2+ speciation in artificial cytoplasms. A key challenge to studying RNA under *in vivo*-like conditions is knowing how components affect the speciation of Mg2+ between free and chelated. Published binding constants for cellular components can be unreliable, as they typically apply only to solutions with specific ionic character,27 and more often, binding constants are not known at all.22 Lastly, predicting Mg2+ speciation using binding constants requires 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 measured using HQS approximated the free Mg2+ concentration that was calculated using our measured binding constants, when the free Mg2+ was in the biological range of 0.5 to 3 mM. However, the calculation was not accurate at higher free Mg2+ concentrations where interactions of Mg2+ with more than one metabolite became likely (Figure 1 E-G). Thus, the HQS assay provided invaluable information on Mg2+ speciation in biologically-relevant solutions, without requiring assumptions or Mg2+ binding constants and interaction coefficients among the many metabolites. Although we used this assay to directly measure Mg2+ speciation in mixtures of metabolites, it could be applied to Mg2+ interactions with other biological molecules.

Our Mg2+ speciation calculations and HQS experiments indicated that metabolites play an important role in buffering the free Mg2+ concentration in cells. Recent theoretical and experimental studies have demonstrated that the cellular environment buffers the concentration of biological molecules, effectively reducing concentration noise *in* *vivo*.52,53 In our system, single-site-Mg2+ interactions in Eco80 buffers the free Mg2+ concentration between just 0.5 and 3 mM Mg2+, in the presence of a large total Mg2+ change between 20 and 40 mM Mg2+. This buffering effect was exaggerated at high total Mg2+ concentrations in Eco80, where an increase in the total Mg2+ concentration to an astounding 200 mM increased the free Mg2+ concentration to only ~10 mM.

Our thermodynamic analysis of RNA helices in Eco80 indicated that the *E. coli* metabolome had a net destabilizing effect on RNA helices of about +0.69±0.12 kcal/mol, with destabilizing effects dominating for NTPCM at about +0.48±0.12 kcal/mol and a mixture of destabilizing and stabilizing effects observed for WMCM averaging at about +0.26±0.2 kcal/mol(Figure 2D). This apparently small, +0.69 kcal/mol, destabilizing effect in Eco80 on RNA helices could have beneficial effects on the transcriptome *in vivo*. First, short, marginally-stable helices would not form, leading to accessible RNA regions that could interact with proteins and regulatory small RNA. In contrast, stable RNA secondary structures would still form functional structures. Second, global destabilization of RNA helices should also lead to destabilization of kinetic traps in RNA folding pathways *in vivo*, such as the misfolds observed for the *Tetrahymena ribozyme*.54

Weakening of RNA helix stability in Eco80 can be understood using a model that combines established effects of polar small molecules and Mg2+ on RNA helix stability. Polar small molecules are known to interact favorably with the exposed bases in the unfolded state (Figure 5B).55–58 Likewise, Mg2+ is known to interact favorably with the high density of negative charges in helical RNA. Thus, metabolites may destabilize helices by favoring the unfolded state and Mg2+ stabilizes helices by favoring the helical state (Figure 5C). The changes in helix formation energy in Mg2+-metabolite mixtures demonstrate a balance between metabolites favoring the unfolded state and Mg2+ favoring the helical state (Figure 5C, right). 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, which lead 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. This leads to the inconsistent destabilization and even stabilization of RNA helices observed in WMCM (Figure 2D), dependent on the relative strength of stabilizing Mg2+-RNA interactions and destabilizing metabolite-RNA interactions (Figure 5C).

NTPCM destabilized AU-rich helices more than GC-rich helices (SI Figure 4). A similar destabilizing effect on RNA G-quadruplex structures has been reported specifically for cytidine nucleotides.59 Interestingly, in the case of G-quadruplex structures, other nucleotides (A and U) had a smaller destabilizing effect, suggesting that G-quadruplexes are destabilized by favorable base-pairing interactions between cytidine nucleotides in solution and Gs in the unfolded state of the RNA. The NTPCM is comprised mostly of ATP, UTP, and dTTP (22.5 mM total versus 4.9 mM GTP). The ATP, UTP, and dTTP are expected to form stronger hydrogen bonds with Us and As, respectively, in the unfolded state of RNA, supporting the AU dependence of helix destabilization by NTPCM.

Map

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Figure 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. The average number of molecules (colored sphere models) in Eco80 that would occupy a sphere with a 50 Å radius were placed randomly around an 8 base-pair RNA helix using Pymol (blue cartoon, PDB 1SDR). Mg2+ ions are represented with teal spheres. Solvent (red wires) and K+ (blue spheres) where modeled using WAXSiS.46 (B-C) Mechanism for destabilization of helices by metabolites and stabilization of helices by Mg2+. Net effect of metabolite-chelated Mg2+ combines metabolite interactions (red, white, blue) favoring the unfolded state and Mg2+ interactions (green) favoring the helical state. (D-E) In-line degradation of the RNA backbone mediated by Mg2+ hydroxide species is inhibited by Mg2+ chelation.

Our analysis of RNA degradation in Eco80 indicated that metabolites protect susceptible regions of RNA 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 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 rates were weakly reduced by depletion of active Mg2+-OH- species in the presence of weak Mg2+ chelators and strongly reduced by depletion of active Mg2+-OH- species in the presence of strong Mg2+ chelators (Figure 5E).

Our analysis of CPEB3 catalysis in Eco80 indicated that 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+ had no effect on reaction rates.24 Similarly, our results in metabolite mixtures found that WMCM weakly enhanced CPEB3 ribozyme catalysis while NTPCM weakly inhibited CPEB3 ribozyme catalysis. A previous study of the CPEB3 ribozyme in the presence of weak amino acid-chelated Mg2+ indicated that rate enhancement was not driven by direct interactions between amino acid-chelated Mg2+ and the catalytic site, but by stabilization of catalytically relevant CPEB3 ribozyme structure.23 In contrast, we observed thermodynamic destabilization of helices and reduction of CPEB3 catalysis in Eco80 and NTPCM, indicating that reduction in catalysis was caused by destabilization of the catalytically relevant structure. Thus, ribozyme rate enhancement *in vivo* is likely dependent on the presence of weak Mg2+ chelators that stabilize the catalytically relevant structure and depletion of strong Mg2+ chelators that destabilize the catalytically relevant structure.

Eco80 had opposing effects on the thermodynamic and chemical stabilities of RNA, which reflected 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 using the speciation of Mg2+ between weak and strong Mg2+-metabolite complexes. The effects of Eco80 reflect RNA function *in vivo*, enhance the biological relevance of mechanistic studies of RNA, and suggest possible ways to enhance the storage of mRNA vaccines.

ASSOCIATED CONTENT

**Supplemental Information**. Supplemental methods, Supplemental figures, Supplemental tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

RNA, ribonucleic acid; DNA, deoxynucleic acid; Mg2+, divalent magnesium ion; HQS, 8-Hydroxy-5-quinolinesulfonic acid; Eco80, 80% of *E. coli* metabolites; NTPCM, nucleotide triphosphate-chelated Mg2+; WMCM, weak metabolite-chelated Mg2+; ITC, isothermal titration calorimetry; SAXS, small angle X-ray scattering; ILP, in-line probing.

REFERENCES

(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) Morandi, E.; van Hemert, M. J.; Incarnato, D. SHAPE-Guided RNA Structure Homology Search and Motif Discovery. *Nat. Commun.* **2022**, *13* (1), 1722. https://doi.org/10.1038/s41467-022-29398-y.

(6) 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.

(7) 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.

(8) 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.

(9) 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.

(10) 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.

(11) 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.

(12) 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.

(13) 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.

(14) 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.

(15) 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.

(16) 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.

(17) 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.

(18) 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.

(19) 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.

(20) 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.

(21) 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.

(22) 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.

(23) 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.

(24) 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.

(25) 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.

(26) 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.

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

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

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

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

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

(32) 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.

(33) 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).

(34) 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.

(35) 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.

(36) 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.

(37) 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.

(38) 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.

(39) 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.

(40) 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.

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

(42) 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.

(43) 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.

(44) 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.

(45) 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.

(46) 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.

(47) Grant, T. D. Ab Initio Electron Density Determination Directly from Solution Scattering Data. *Nat. Methods* **2018**, *15* (3), 191–193. https://doi.org/10.1038/nmeth.4581.

(48) Chen, J.-H.; Yajima, R.; Chadalavada, D. M.; Chase, E.; Bevilacqua, P. C.; Golden, B. L. A 1.9 Å Crystal Structure of the HDV Ribozyme Precleavage Suggests Both Lewis Acid and General Acid Mechanisms Contribute to Phosphodiester Cleavage. *Biochemistry* **2010**, *49* (31), 6508–6518. https://doi.org/10.1021/bi100670p.

(49) Chadalavada, D. M.; Gratton, E. A.; Bevilacqua, P. C. The Human HDV-like *CPEB3* Ribozyme Is Intrinsically Fast-Reacting. *Biochemistry* **2010**, *49* (25), 5321–5330. https://doi.org/10.1021/bi100434c.

(50) Robertus, J. D.; Ladner, J. E.; Finch, J. T.; Rhodes, D.; Brown, R. S.; Clark, B. F. C.; Klug, A. Structure of Yeast Phenylalanine TRNA at 3 Å Resolution. *Nature* **1974**, *250* (5467), 546–551. https://doi.org/10.1038/250546a0.

(51) 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.

(52) 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.

(53) 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.

(54) Mitchell, D.; Russell, R. Folding Pathways of the Tetrahymena Ribozyme. *J. Mol. Biol.* **2014**, *426* (12), 2300–2312. https://doi.org/10.1016/j.jmb.2014.04.011.

(55) 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.

(56) 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.

(57) 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.

(58) 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.

(59) 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.