ABSTRACT: Herein, we examine the complicated network of interactions among RNA, the metabolome, and the metalome in conditions that mimic the *E. coli* cytoplasm. First, 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. Then, we used this information to inform creation of artificial cytoplasms that mimic *in vivo E. coli* conditions, termed Eco80. We empirically determined that the mixture of *E. coli* metabolites in Eco80 approximates single sit binding behavior towards Mg2+ in the biologically relevant free Mg2+ range of ~0.5 to 3 mM Mg2+, using a Mg2+ binding fluorescent dye (8-Hydroxy-5-quinolinesulfonic acid). Furthermore, we examined the effects of Eco80 conditions on the thermodynamic stability, chemical stability, catalysis, and compactness of RNA. We find that these Eco80conditions lead to opposing effects, wherein thermodynamic stability of RNA helices were weakened but chemical stability, compactness, and catalysis were enhanced. We propose a mechanism where increased RNA compactness and catalysis is facilitated in Eco80.

**Introduction**

Summary of progress on in vivo-like conditions.

Studies that consider cellular components one at a time.

Studies that consider cellular components together in artificial cytoplasm.

In contrast, 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 relavent concentrations of monovalent ions and free Mg2+ ions.This *aufbau* approach allows us to understand the effects of 80% of metabolite and metal ion species that compose the network of interactions that RNA experiences in *E. coli* cells.

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

*E. coli* cells contain hundreds of metabolites (about 243 mM total), which is too many metabolites to test systematically. However, 15 abundant metabolites, an experimentally manageable number, comprise 80% (195 mM) of total metabolites (Figure 1A). Thus, we sought to prepare Eco80*,* an artificial cytoplasm containing the biological concentrations of the 15 most abundant metabolites in *E. coli* (Table 1).

All of the metabolites in Eco80 are zwitter ions or negatively charged near physiological pH (~7) and require electrostatic neutralization with metal ions. Thus, we prepared Eco80 so that the final monovalent ion concentration was the physiological value of 240 mM Na+ and 140 mM K+ (Supplementary information (SI) Table 1). 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 was added to a final 240 mM Na+ and 140 mM K+. We thus created Eco80, at a 2x final concentration so that it could be diluted into other reagents for experiments.

Next, we considered how metabolites effect the speciation of free and chelated Mg2+. All 15 Eco80 metabolites have functional groups, carboxylates and phosphates, that drive chelating interactions with divalent 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. While extensive literature exists on chelating interactions between Mg2+ and small molecules, our previous estimates are putative as Mg2+ chelation strength is dependent on environmental factors such as pH, ionic strength, the composition of background ions, and temperature. Thus, we sought to better characterize Mg2+ chelation by the metabolites inEco80,at the physiological background of 240 mM Na+, 140 mM K+, pH 7.0, and 37 °C.

We determined apparent disassociation constants (KD) for Eco80 metabolites in 240 mM NaCl, 140 mM KCl, pH 7.0 buffer at 37 °C (Table 1). Isothermal titration calorimetry (ITC) was used to determine KDs for phosphorylated metabolites (SI figure 1, SI Table 2). A fluorescence assay, that measures the free Mg2+ concentration in a sample using the metal ion binding dye 8-Hydroxy-5-quinolinesulfonic (HQS) acid, was used to estimate the KD for Mg2+ metabolites that did not produce enough heat on binding to measure with ITC (SI figure 2, SI Table 3). For the HQS assay, Mg2+ is titrated into HQS solutions in the absence and presence of chelators. HQS emission as a function of the total Mg2+ in the absence of chelators is then fit to a binding model (SI figure 2A, top blue data and black fit). The free Mg2+ concentration is then then calculated from the fluorescence emission for each data point using the binding model, providing the free Mg2+ concentration as a function of the total Mg2+ concentration (SI figure 2B, bottom). Mg2+ binding by metabolites is thus observed by fitting the free Mg2+ concentration as a function of the total Mg2+ concentration, which is shifted to the right as Mg2+ is sequestered by metabolites.

The four nucleotide triphosphates, ATP, UTP, GTP, and dTTP, were classified as strong Mg2+ binders, with KD values less than the approximate free Mg2+ concentration in *E. coli,* 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, dihydroxyacetone phosphate, and pyruvic acid were classified as weak Mg2+ binders, with a KD value 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). We thus broke Eco80 down into two other artificial cytoplasms, NTP chelated Mg2+ (NTPCM), and weak metabolite chelated Mg2+ (WMCM), composed of the strong Mg2+ chelators (nucleotide triphosphates), and weak Mg2+ chelators, respectively (Table 1).

We next used two methods to estimate how the metabolites effect the speciation of free and chelated Mg2+ as a mixture. The first method was experimental, using HQS emission to estimate the free Mg2+ concentration in the presence of metabolites (Figure 1B-C, SI Table 3). The second method was a statistical model that accounts for experimental uncertainties in metabolite concentrations and uncertainty in KD determination, based on single-site binding (meaning that one metabolite associates one Mg2+). 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 to create 1000 virtual artificial cytoplasms, 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 a metabolite in a mixture, and KD is the disassociation constant.

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

The two methods indicate that Mg2+ speciates in artificial cytoplasms according to a single-site model within or below the biological free Mg2+ range of 0.5 to 3 mM Mg2+, but not at higher free Mg2+ concentrations (Figure 1 E-F). For example, in Eco80, the statistical model suggests that the free Mg2+ should increase slowly as the total Mg2+ concentration is increased, until the strong Mg2+ chelators (NTPs) become saturated at about 27 mM total Mg2+ (Figure 1E, hex bins). At total Mg2+ concentrations higher than 27 mM, the free Mg2+ should increase faster because the NTPs are saturated by Mg2+ and the weak chelators sequester less Mg2+. Free Mg2+, calculated using HQS emission shows a similar trend to the statistical model below 3 mM Mg2+ free (Figure 1E, data points). However , the free Mg2+ concentration calculated from HQS emission does not increase with the total Mg2+ as fast as the single-site model would predict above 3 mM free Mg2+, indicating that multivalent interactions, where one metabolite interacts with several Mg2+ molecules, dominate the equilibrium. Non-single-site behavior above 3 mM free Mg2+ is also observed in the NTPCM and WMCM artificial cytoplasms (Figure 1 F & G).

Lastly, we sought to empirically determine how much total Mg2+ is required to maintain 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 was fit to a polynomial to empirically approximate the data (Figure 1 E-G, colored lines), and the total Mg2+ concentration required to produce 2 mM Free Mg2+ was calculated from the polynomial fit. This resulted in a 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).

**Eco80 destabilizes RNA helices**

*Paragraph 1: Transition*

We sought to understand how Eco80 effects the stability of RNA helices composed of Watson-Crick base pairs. RNA helix stability is traditionally measured with UV-absorbance melting curves, usually at 260 or 280 nm, which allows for accurate calculation of helix folding energies. However, absorbance melting curves were not appropriate for measuring helix stability in Eco80 because of the high absorbtivity of ATP, UTP, GTP, dTTP, and UDP-N-acetylglucosamine. Thus, we used a fluorescence binding isotherm assay, which is optically orthogonal to Eco80, to accurately determine helix folding energies in Eco80.

Paragraph 2: Fluorescence isotherms-intro

Helix stability was measured using the emission of a 5’-fluorophore (FAM-RNA) labeled RNA strand in equilibrium with a complementary 3’-quencher (RNA-BHQ1) labeled RNA strand (Figure 2A). High emission indicates that the FAM-RNA is single-stranded and low emission indicates that the FAM-RNA is bound in duplex with a RNA-BHQ1 strand (Figure 2A). We used a binding isotherm method, where RNA-BHQ1 is titrated into a constant concentration of FAM-RNA (SI figure 3), resulting in an apparent binding isotherm, with high FAM-RNA emission at 0 or low RNA-BHQ1 concentrations and low FAM-RNA emission at high RNA-BHQ1 concentrations. Fluorescence binding isotherms were favored over fluorescence melts with equal molar concentrations of FAM-RNA and RNA-BHQ1 because of the unpredictable dependence of FAM emision at different temperatures. FAM-emission 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). Fluorescence isotherms were fit at each temperature to Supplemental Equation X, thus determining the KD between the FAM-RNA strand and the RNA-BHQ1 strand.

Paragraph 3: MeltR

Raw fluorecence was fit with a new program called MeltR, created by the authors, to determine folding energies. MeltR is a package of functions for the popular R programming language that allow facile conversion of raw data to folding energies. Importantly, MeltR handles two sources of experimental error: (1) uncertainties in determined RNA concentration and (2) inaccurate KDs collected at low and high temperatures.

Paragraph 4-6: MeltR concentration optimization algorithm

We found that helix energies from fitting fluorescence binding isotherms are highly dependent on the determination of labeled RNA concentrations, which is uncertain because of extinction coefficient uncertainty, but errors are propagated systematically (SI Figure 3). To understand why, we modeled data assuming a folding enthalpy (ΔH), entropy (ΔS), and Gibb’s free energy at 37 °C (ΔG) of -56.2 kcal/mol, -136. 4 cal/mol/K, and -13.9 kcal/mol respectively, 5% random fluorescence error, perfectly accurate determination of RNA concentrations in concentrated stocks (a 200 nM FAM-RNA concentration, and 0, 1, 10, 50, 100, 150, 200, 250, 400, 600, 800, and 1000 nM RNA-BHQ1 concentrations). We then used MeltR to fit the modeled, resulting in accurate determination of ΔH = -56.0 kcal/mol, ΔS = -135.7 cal/mol/K, and ΔG = -13.9 kcal/mol. We next considered how assuming incorrect RNA stock concentrations could effect the accuracy of the fits. Error (-50% to +50%) was seeded into virtual stock concentrations and the data were refit (SI figure 1 A, “none”). We found that fit accuracy what highly dependent on error in stock concentrations, unless the FAM-RNA error and the RNA-BHQ1 error compensated for each other, e.g. %FAM-RNA error = %FAM-BHQ1 error.

We next considered a more realistic scenario, where the experiment assumes perfectly accurate determination of RNA concentrations but there is actually +20% FAM-RNA concentration error. Data was modeled using the same folding energies and RNA-BHQ1 concentrations, but with a 240 nM FAM-RNA concentration. We then used MeltR to fit the modeled data, assuming a 200 nM FAM-RNA concentration, resulting in inaccurate determination of H = -35.8 kcal/mol, ΔS = -75.5 cal/mol/K, and ΔG = -12.3 kcal/mol. Once again, we considered how assuming incorrect RNA stock concentrations could effect the accuracy of the fits. Error (-50% to +50%) was seeded into virtual stock concentrations and the data were refit (SI figure 1 B). Similar to SI figure 1 A, we found that fit accuracy what highly dependent on error in stock concentrations. However, the fits were most accurate according to Equation 1 instead of where %FAM-RNA error = %FAM-BHQ1 error:

Where X is the actual FAM-RNA concentration divided by the estimated FAM-RNA concentration (240 nM/200 nmol = 1.2 in this example) Thus, MeltR does not need perfectly accurate concentrations, just an optimization algorithm that finds the FAM-RNA concentration correction factor termed X. To find X, MeltR selects an isotherm (usually the lowest temperature), where the KD is more than 10 times less than the FAM-RNA labeled concentration (SI figure 4 D). At this KD range, the shape of the binding curve is independent of KD, and MeltR uses the isotherm as a Job plot to determine X. MeltR uses X to correct the FAM-RNA concentration. We next tested the MeltR optimization algorithm. The modeled data, assuming perfectly accurate determination of RNA concentrations when there is actually a +20% FAM-RNA concentration error, was fit using MeltR with the concentration optimization algorithm, resulting in a more accurate determination of ΔH = -51.9 kcal/mol, ΔS = -123.5 cal/mol/K, and ΔG = -13.6 kcal/mol. We then seeded addional error into the data set (-50% to +50%), refit the data using the MeltR concentration optimization algorithm, and found that MeltR calculates accurate folding energies (within 0.2 kcal/mol in terms of the ΔG on average) even with large inaccuracies in reagent concentration estimates (SI Figure 4C).

Paragraph 5: MeltR only fits data from the most accurate isotherms

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Paragraph 6: Helix design

Paragraph 7: Results

**Eco80 protects RNA from degradation**