Main text outline

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 10 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 an artificial cytoplasm that contains 80% of *E. coli* metabolites with biological concentrations of monovalent ions and free Mg2+ ions.This aufbau approach allows us to understand the effects of most of the metabolite and metal ions 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 for a total metabolite concentration of about 243 mM, too many metabolites to test systematically . However, 15 abundant metabolites comprise 80% (194 mM) of total metabolites, and 15 metabolites is an experimentally manageable number (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 charged near physiological pH (~7) and require electrostatic neutralization with metal ions. Thus, we prepared Eco80 so that the final monovalent ion concentration was close to the physiological value of 240 mM Na+ and 140 mM K+ (SI Table 1). Metabolite salts and free acids were prepared to a final 2xconcentration in volumetric flasks, 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+.

Next, we considered how metabolites effect the speciation of free and chelated Mg2+. All 15 of the most abundant 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+, an ionic strength of 0.15 M and a pH of 7.5. While extensive liturature exists on chelating interactions between Mg2+ and small molecules, our previous estimates are putative as Mg2+ chelation strength is dependent on enviromental factors such as pH, ionic strength, composition of background ions, and temperature. Thus, we sought to better characterize the Mg2+ chelation by the top 15 most abundant *E. coli* metabolites at the physiological background of 240 mM Na+, 140 mM K+, pH 7.0, and 37 °C.

Isothermal titration calorimetry (ITC) was used to determine disassociation constants (KD) for 9 of the 15 most abundant *E. coli* metabolites in 240 mM NaCl, 140 mM KCl, 10 mM HEPES pH 7.0 buffer at 37 °C. Weaker Mg2+ binders,