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Zn(II) binding to *E. coli* 70S Ribosomes

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Abstract

E. coli 70S ribosomes tightly bind 8 equivalents of Zn(II), and EXAFS spectra indicate that Zn(II) may be protein-bound. Ribosomes were incubated with EDTA and Zn(II), and after dialysis, the resulting ribosomes bound 5 and 11 Zn(II) equivalents, respectively. EXAFS studies show that the additional Zn(II) in the zinc-supplemented ribosomes bind in part to the phosphate backbone of ribosome. Lastly, *in vitro* translation studies demonstrate that EDTA-treated ribosomes do not synthesize an active Zn(II)-metalloenzyme, while the as-isolated ribosomes do. These studies demonstrate that the majority of intracellular Zn(II) resides in the ribosome.

Keywords

E. coli; ribosomes; zinc

Zinc is an essential transition metal, required for life in all organisms[2]. It plays key catalytic roles in enzymes from all six major classes[3], as well as a structural role in numerous transcriptional activators and regulators[4, 5]. While Zn(II) import and export is well understood[2, 6–12], surprisingly little is known about the fate of intracellular zinc. The free Zn(II) concentration within a cell has been estimated to be in the femtomolar range, while the total cellular concentration has been established as approximately 200 μM[13]. However, only about 12% of the cellular Zn(II) has been accounted for as bound to Zn(II) metalloproteins[13], leaving open the question as to where the remaining Zn(II) resides in the cell.

Previous studies have suggested that Zn(II) is associated with the ribosome. Atomic absorption spectroscopy of *E. coli* 70S ribosomes revealed 2 equivalents of bound Zn(II)[14], while a PAR assay of ribosomes from *B. subtilis* indicated 2.5 eq of closely-associated Zn(II)[15]. Thus, while an association of zinc with the ribosome has been indicated previously, the amount of metal present in active ribosomes has not been accurately determined. Further, it has yet to be established whether ribosomal Zn(II) remains associated with the ribosome at all times or is labile. We report here biophysical studies that show strong and weak binding Zn(II) to the ribosome.

E. coli 70S ribosomes were isolated and quantified as described previously[16]. To verify that the isolated ribosomes were intact and functional, *in vitro* transcription/translation assays were performed using the PURESYSTEM Classic II mini alpha kit[17] and plasmid pUB5830[18], which contains the gene for metallo-β-lactamase L1, which binds two Zn(II) ions[18], from *Stenotrophomonas maltophilia*. After *in vitro* transcription/translation, the

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Supporting Information Available: Detailed experimental procedures and a description of the XAS data analysis, including one Figure and one Table, are provided as Supplementary Material. This material is available free of charge at http://pubs.acs.org.

reaction mixture was assayed using nitrocefin as substrate, which indicated the production of 9.7 ± 1.9 μg of L1. Control reactions, conducted in the absence of ribosomes, did not generate metallo-β-lactamase activity, demonstrating the viability of some of the isolated ribosomes. *In vitro* transcription/translation experiments using the kit-provided, non-Zn(II) binding dihydrofolate reductase (DHFR) gene from *E. coli* were also conducted (see Supporting Information). These assays revealed that 0.072 ± 0.014 μg of DHFR were produced. Using the ICP-MS of the purified *E. coli* 70S ribosomes showed 8 equivalents of Zn(II) (Table 1). Only trace amounts of other transition metal ions, such as Co, Cu, Mn, Ni, and Fe, were present.

To examine the minimum and maximum Zn(II) content of the *E. coli* 70S ribosomes, ribosome samples were incubated with up to 100 eq of Zn(II) to fully populate weak binding sites, or up to 40 eq of EDTA to fully de-populate them, followed by exhaustive dialysis against lysis buffer (see Supporting Information). The concentration of Mg(II) was maintained at 10 mM throughout the incubation and dialysis steps and also during all *in vitro* transcription/translation assays since previous studies have suggested that ribosomes do not “fall apart” as long as the Mg(II) concentration does not fall under 5–7 mM[19–21]. The Zn(II)-supplemented ribosomes bound 11 eq of Zn(II), while the EDTA-treated ribosomes were found to contain only 5 eq of Zn(II) (Table 1), indicating 5 tight-binding Zn(II) sites and up to 6 weaker Zn(II) binding sites.

In vitro transcription/translation reactions were conducted on the Zn(II)-supplemented and EDTA-treated ribosomes, which were subjected to similar dialysis conditions, to determine if the treated ribosomes were active. Reactions with Zn(II)-supplemented ribosomes produced 5.4 ± 1.1 μg of L1 and 0.062 ± 0.012 μg of DHFR. EDTA-treated ribosomes produced no detectable L1 but produced 3.5 ± 0.6 μg of DHFR. The higher amounts of DHFR, as determined from activity assays, produced in the EDTA-treated reactions is probably due to inhibition of DHFR activity by mono- and divalent metal cations[22]. The production of no L1 in EDTA-treated reactions suggests a role of Zn(II) in the *in vitro* transcription/translation of this Zn(II)-metalloenzyme. Future studies will further address the role of Zn(II) in ribosome activity; nonetheless, these studies demonstrate that the EDTA/Zn(II) treatments and dialysis steps did not result in inactive ribosomes.

The nature of the Zn(II) binding sites were investigated by acquiring EXAFS spectra for Zn(II)-supplemented and EDTA-treated ribosomes (labeled as +Zn and −Zn in Figure 1, respectively). The two samples showed similar spectra (Figure 1, top), with the main peaks in their Fourier transforms nearly superimposable. Both samples show a single prominent outer shell feature, which moves to lower R in the Zn(II)-supplemented data. Fits to the data for EDTA-treated ribosomes, representing the average of the tight binding sites, indicate a primary coordination sphere of 1 N/O donor at 1.93 Å, 3 N/O donors at 2.11 Å, and 1 sulfur donor at 2.34 Å. The outer shell feature is best modeled as a shell of 3 carbon scatterers at 3.25 Å, presumably from carboxylate ligands (Figure 1, center, and Figure S1). Any attempt to model this feature with phosphorus, as might be anticipated if the ribosomal Zn(II) were interacting with the phosphate backbone of the ribosome’s constitutive RNA, was unsuccessful. In comparison, the EXAFS data for Zn(II)-supplemented ribosomes were best fit with a similar first shell of 2 N/O ligands at 1.96 Å, 3 N/O ligands at 2.14 Å and 0.5 sulfur donors at 2.29 Å. The outer shell feature, which shifts to $R + \alpha = 2.5$ Å (from 2.9 Å in the Zn(II)-depleted data), is best modeled with a mixture of 3 carbon scatterers at 3.15 Å and 1.5 phosphorous at 2.97 Å (Figure 1, bottom, and Figure S2). Both contributions appear warranted, as inclusion of the carbon shell alone leads to a 3-fold improvement in fit residual, while inclusion of the phosphorus shell alone results in more than 4-fold improvement in the fit. Inclusion of both leads to a nearly 9-fold reduction in fit residual, and their refined distances are separated by more than the 0.16 Å resolution of the data.

Thus, analysis of the XAS data indicates that the Zn(II) that is tightly associated with the ribosome (EDTA-treated) is most likely protein bound although we cannot rule out Zn(II) binding to non phosphate ligands of RNA that are buried or regions of interface between protein and RNA non phosphate ligands. However, there is at least some fraction of the more loosely associated Zn(II) (Zn(II)-supplemented), which interacts directly with the phosphate backbone of RNA.

The number of ribosomes present in an *E. coli* cell is, not surprisingly, dependent upon its growth stage. The number of ribosomes present has been estimated to fluctuate from 2,000 per cell at low growth to 70,000 at rapid growth[23]. Given the average volume of an *E. coli* cell, approximately 1.8 femtoliters,[13] the present studies allow the total ribosomal Zn(II) content to be estimated. Using the as-isolated value of 8 equivalents of Zn(II) per ribosome, the concentration of Zn(II) contained within ribosomes is on average 20 μM at low growth, and 0.52 mM under rapid growth conditions. Estimates of total cellular Zn(II) range from 0.2 mM to 0.8 mM[13, 24]. Thus, the present studies suggest that a large portion of cellular Zn(II) is contained in the ribosome.

Previous studies have suggested as many as eight ribosomal proteins that are capable of binding Zn(II) (20, 22). The *B. subtilis* ribosomal protein L31 has clearly been shown to bind Zn(II)[25]. The solution structure of ribosomal protein L36 from *T. thermophilus* revealed a Zn(II)-ribbon-like fold[26], suggesting it, too, may bind Zn(II). However, none of the available crystal structures show Zn(II) bound to L31 (Hensley, Crowder, unpublished results). Further proteomic studies have suggested that *E. coli* ribosomal proteins L2, L13, S2, S15, S16, and S17 could bind Zn(II) (Figure 2)[27]. None of these proteins are in close proximity to the peptide exit site and none, except L36[28], have been shown to bind Zn(II) in current crystal structures of ribosomes (Hensley, Crowder, unpublished results). This result may be due to the procedure used to purify ribosomes[16], which contains EDTA, or to misidentification of Zn(II) as a Mg(II) in some of the early crystal structures. Our finding that 8 Zn(II) are bound to the ribosome under normal conditions indicates that perhaps all of the proteomics-identified proteins bind Zn(II).

The current report supports the possible presence of four Zn(II) binding proteins in the large ribosomal subunit of the 70S *E. coli* ribosome (L36, L31, L13, and L2) and four Zn(II) binding proteins in the small subunit (S17, S16, S15, and S2) (Figure 2). Ribosome-associated Zn(II) identified in this study could possibly serve in a structural (non-catalytic) role for the ribosomal proteins. Further study on these potential Zn(II) binding proteins is required to validate that these proteins bind Zn(II). Currently, investigations by our group into the metal binding capabilities of recombinant *E. coli* L36, L31, and L13 have indicated all these proteins are able to bind Zn(II) (M. P. Hensley and M. W. Crowder, unpublished). Future studies will focus identifying the remaining Zn(II)-binding, ribosomal proteins and probing their physiological role(s).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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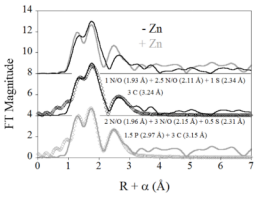

Figure 1.

Comparison of the EXAFS Fourier transforms for Zn(II)-depleted (−Zn, solid black line) and Zn(II)-supplemented (+Zn, solid gray line) *E. coli* ribosomes (top), and the corresponding best fits (open symbols, see Table S1 for details).

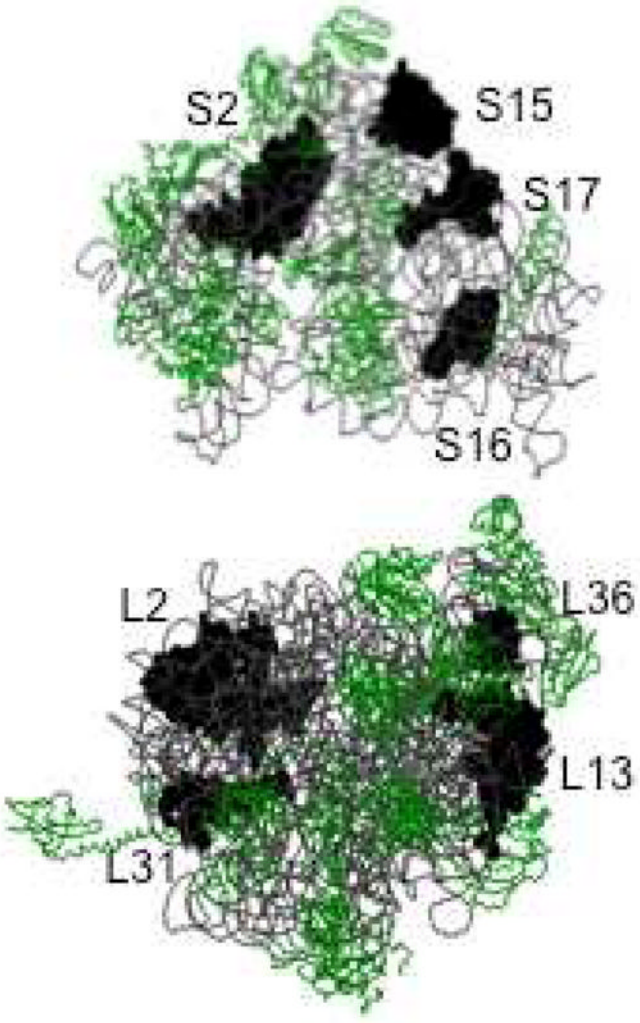

Figure 2.
Structure of *E. coli* 30S (top) and 50S (bottom) ribosome with potential Zn(II) binding proteins labeled. This figure was rendered with Raswin using Protein Databank coordinates 2AVY and 2AW4[1].

Table 1

Metal content of *E. coli* 70S ribosomes.

samples	Zn(II) content (eq/ribosome)	other metals (Co,Cu,Mn,Ni,Fe)
as-isolated	7.9 ± 0.1	0.5 ± 0.3
Zn(II)-supplemented	11 ± 1	0.8 ± 0.3
Zn(II)-depleted	5.0 ± 0.1	0.8 ± 0.3