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# Zn(II) Binding to *Escherichia coli* 70S Ribosomes

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Supporting Information

**ABSTRACT:** *Escherichia coli* 70S ribosomes tightly bind 8 equiv 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 equiv of Zn(II), respectively. EXAFS studies show that the additional Zn(II) in the zinc-supplemented ribosomes binds in part to the phosphate backbone of the ribosome. Lastly, in vitro translation studies demonstrate that EDTA-treated ribosomes do not synthesize an active Zn(II)-bound metalloenzyme, while the as-isolated ribosomes do. These studies demonstrate that the majority of intracellular Zn(II) resides in the ribosome.

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 are 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 to be approximately 200 μM.[13] However, only ~12% of the cellular Zn(II) has been identified as being bound to Zn(II) metalloproteins,[13] leaving open the question of 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 *Escherichia coli* 70S ribosomes revealed 2 equiv of bound Zn(II),[14] while a PAR assay of ribosomes from *Bacillus subtilis* indicated 2.5 equiv 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 of 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 and translation, the reaction mixture was assayed using nitrocefin as a 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 the Supporting Information). These assays revealed that 0.072 ± 0.014 μg of DHFR was produced. ICP-MS of the purified *E. coli* 70S ribosomes showed 8 equiv of Zn(II) (Table 1). Only trace amounts of other transition metal ions, such as Co, Cu, Mn, Ni, and Fe, were present.

**Table 1. Metal Content of *E. coli* 70S Ribosomes**

| sample | Zn(II) content (equiv/ribosome) | content of other metals (Co, Cu, Mn, Ni, Fe) (equiv) |
|---|---|---|
| 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 |

To examine the minimum and maximum Zn(II) content of the *E. coli* 70S ribosomes, we incubated ribosome samples with up to 100 equiv of Zn(II) to fully populate weak binding sites or up to 40 equiv of EDTA to fully depopulate them, followed by exhaustive dialysis against lysis buffer (see the 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 because 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 equiv of Zn(II), while the EDTA-treated ribosomes were found to contain only 5 equiv of Zn(II) (Table 1), indicating five tight binding Zn(II) sites and up to six weaker binding Zn(II) 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 larger amounts of DHFR, as determined from activity assays, produced in the EDTA-treated reactions are 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

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transcription and translation of this Zn(II)-bound metalloenzyme. Future studies will further address the role of Zn(II) in ribosome activity; nonetheless, these studies demonstrate that the EDTA and Zn(II) treatments and dialysis steps did not result in inactive ribosomes.

The nature of the Zn(II) binding sites was 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 being nearly superimposable. Both samples show a single prominent outer shell feature, which moves to a 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 one N/O donor at 1.93 Å, three N/O donors at 2.11 Å, and one sulfur donor at 2.34 Å. The outer shell feature is best modeled as a shell of three carbon scatterers at 3.25 Å, presumably from carboxylate ligands (Figure 1, center, and Figure S1 of the Supporting Information). 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 two N/O ligands at 1.96 Å, three N/O ligands at 2.14 Å, and one-half sulfur donor at 2.29 Å. The outer shell feature, which shifts to an $R + \alpha$ values of 2.5 Å [from 2.9 Å in the Zn(II)-depleted form], is best modeled with a mixture of three carbon scatterers at 3.15 Å and 1.5 phosphorus atoms at 2.97 Å (Figure 1, bottom, and Figure S2 of the Supporting Information). Both contributions appear warranted, as inclusion of the carbon shell alone leads to a 3-fold improvement in the fit residual, while inclusion of the phosphorus shell alone results in a more than 4-fold improvement in the fit. Inclusion of both leads to a nearly 9-fold reduction in the 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 the possibility that Zn(II) binds 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.

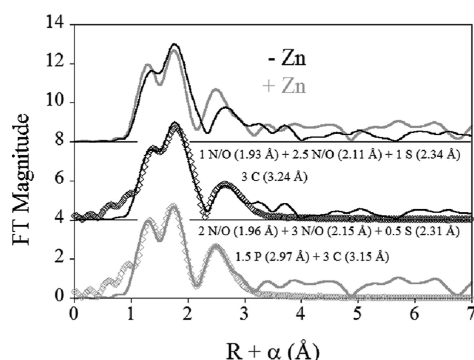


**Figure 1.** Comparison of the EXAFS Fourier transforms for Zn(II)-depleted (−Zn, solid black line) and Zn(II)-supplemented (+Zn, solid gray line) *E. coli* ribosomes (top), and the corresponding best fits (empty symbols; see Table S1 of the Supporting Information for details).

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 2000 per cell at low rates of growth to 70000 at rapid rates of growth.[23] Given the average volume of an *E. coli* cell, approximately 1.8 fL,[13] these studies allow the total ribosomal Zn(II) content to be estimated. Using the as-isolated value of 8 equiv of Zn(II) per ribosome, the concentration of Zn(II) contained within ribosomes is on average 20 $\mu$M at low rates of growth and 0.52 mM under rapid growth conditions. Estimates of the total cellular Zn(II) concentration range from 0.2 to 0.8 mM.[13,24] Thus, these 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] *B. subtilis* ribosomal protein L31 has clearly been shown to bind Zn(II).[25] The solution structure of ribosomal protein L36 from *Thermus 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 (M. P. Hensley and M. W. Crowder, unpublished results). Further proteomic studies have suggested that *E. coli* ribosomal proteins L2, L13, S2, and S15−S17 could bind Zn(II) (Figure 2).[27] None of these proteins are in the proximity of the peptide exit site, and none, except L36,[28] have been shown to bind Zn(II) in current crystal structures of ribosomes (M. P. Hensley and M. W. 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 equiv of Zn(II) is bound to the ribosome under normal conditions indicates that perhaps all of the proteomics-identified proteins bind Zn(II).

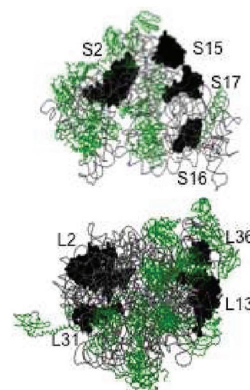


**Figure 2.** Structure of the *E. coli* 30S (top) and 50S (bottom) ribosome with potential Zn(II) binding proteins labeled. This figure was rendered with Raswin using Protein Data Bank entries 2AVY and 2AW4.[1]

This report supports the possible presence of four Zn(II) binding proteins in the large ribosomal subunit of the 70S *E. coli* ribosome (L2, L13, L31, and L36) and four Zn(II) binding proteins in the small subunit (S2 and S15−S17) (Figure 2). Ribosome-associated Zn(II) identified in this study could possibly serve in a structural (noncatalytic) role for the ribosomal proteins. Further study of these potential Zn(II) binding proteins is required to validate that these proteins bind Zn(II). Currently, investigations by our group into the metal


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binding capabilities of recombinant *E. coli* L13, L31, and L36 have indicated all these proteins are able to bind Zn(II) (M. P. Hensley and M. W. Crowder, unpublished results). Future studies will focus on identifying the remaining Zn(II) binding, ribosomal proteins and probing their physiological role(s).

## ■ ASSOCIATED CONTENT

### Supporting Information

Detailed experimental procedures and a description of the XAS data analysis, including one figure and one table. This material is available free of charge via the Internet at http://pubs.acs.org.

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