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Bacillithiol, a new role in buffering intracellular zinc

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SUMMARY

Zinc is a catalytic or structural cofactor of numerous proteins but can also be toxic if cells accumulate too much of this essential metal. Therefore, mechanisms of zinc homeostasis are needed to maintain a low but adequate amount of free zinc so that newly translated zinc-dependent proteins can bind their cofactor without confounding issues of toxicity. These mechanisms include the regulation of uptake and efflux transporters and buffering of the free metal concentration by low molecular weight ligands in the cytosol. While many of the transporters involved in zinc homeostasis have been discovered in recent years, the molecules that buffer zinc have remained largely a mystery. In the new report highlighted by this commentary, Ma et al. provide convincing evidence that bacillithiol, the major low molecular weight thiol compound in *Bacillus subtilis*, serves as an important zinc buffer in those cells (Ma et al., 2014). Their discovery provides an important piece to the puzzle of how zinc buffering occurs in a large number of microbes and provides new clues about the role and relative importance of zinc buffering in all organisms.

It has been estimated that 25–30% of all proteins require bound metal ions for their activity (Dupont et al., 2010; Waldron et al., 2009). Binding of the correct metal cofactor by each of these proteins is critically important to their function. Because proteins are structurally flexible, selectivity of metal binding based on ion size and coordination geometry is often insufficient. Moreover, binding of metals by proteins generally follows the Irving-Williams series in which binding occurs with the following order of preference (from weakest to strongest): $Mg^{2+} < Ca^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+}$, $Cu^{+} > Zn^{2+}$ (Silva and Williams, 2001). Binding by metals at the high end of this order (e.g. Zn^{2+} , Cu^{+}) can readily occur and block binding of correct metals that have a weaker propensity (e.g. Mn^{2+} , Fe^{2+}). Thus, a key question in biochemistry is how do metalloproteins get the right metal cofactor?

One solution to this problem is the metallochaperone. Copper metallochaperones such as Ccs1, Atx1, and CopZ bind Cu^{+} soon after it enters the cell and deliver the metal to specific recipient copper-dependent proteins, e.g. superoxide dismutase, through direct protein-protein interactions and ligand exchange reactions (Robinson and Winge, 2010). Such a pathway prevents the promiscuous Cu^{+} from binding in sites where it shouldn't. Similarly, Fe^{2+} is directly targeted to at least some iron-binding proteins through an analogous mechanism. The PCBP proteins have been found to deliver iron to target proteins ferritin, the HIF prolyl hydroxylase, and deoxyhypusine hydroxylase (Frey et al., 2014; Philpott, 2012). For zinc, in contrast, no metallochaperones have yet been discovered and the mechanism of specific metal delivery to zinc-binding apoproteins is much less clear.

Perhaps the most striking fact about zinc is the large number of proteins that require this metal for function. Using bioinformatics approaches, it has been estimated that ~5% of prokaryotic genes and almost 10% of genes in eukaryotes encode zinc-binding proteins (Andreini et al., 2006). These include proteins in all six general enzyme classes (oxidoreductases, transferases, hydrolases lyases, isomerases, and ligases) as well as structural zinc sites such as C₂H₂ zinc fingers and the C₄ zinc sites found in some ribosomal subunits. The ubiquity of zinc as a cofactor probably stems from two important features of this metal. First, zinc is a strong Lewis acid and so it is useful for many catalytic reactions. Second, zinc is not redox active so it can serve as a stable structural cofactor without disruption of this role by oxidative or reductive stress.

Because zinc proteins are so abundant, cells must maintain sufficient quantities of zinc available for binding by newly translated zinc proteins. On the other hand, free zinc levels need to be kept to a minimum to avoid extensive adventitious binding and toxicity. Cells maintain this delicate balance by a process collectively known as “muffling” (Colvin et al., 2010; Thomas et al., 1991). Muffling includes the regulation of intracellular free zinc by controlling a combination of zinc uptake, zinc efflux, organellar compartmentalization (in eukaryotes), and zinc buffering. While great progress has been made in recent years regarding zinc transporters and their roles in muffling (Eide, 2006; Huang and Tepasamorndech, 2013; Lichten and Cousins, 2009), we still know little about the components of zinc buffering. It is on this topic that the recent work by Ma et al. shines new and important light (Ma et al., 2014).

To put this problem in some perspective, consider that total zinc in cells is remarkably high and ranges from 0.1–0.8 mM. This is true for bacterial cells like *Escherichia coli* and *Bacillus subtilis* and eukaryotic cells such as yeast and mouse fibroblasts (Eide, 2006; Ma et al., 2014; Outten and O’Halloran, 2001). The vast majority of this zinc is tightly bound by zinc metalloproteins and only a very small fraction is found in a pool of labile zinc that is available for binding by newly made proteins. The labile pool can be subdivided between free Zn²⁺, in the form of the hydrated Zn(H₂O)₆²⁺ ion, and zinc bound to buffering agents such as small organic ligands and proteins such as metallothioneins. The amount and nature of the various buffering molecules in a cell determines the level of free zinc relative to the total labile pool. Estimates of free zinc in both prokaryotic and eukaryotic cells have been made in a variety of ways and a relatively narrow concentration range of 0.1–1 nM is emerging as the consensus from a variety of assay methods and different cell types (Colvin et al., 2010; Qin et al., 2013; Vinkenborg et al., 2010; Wang et al., 2011). In addition, the measured zinc binding affinities of zinc-sensing regulatory proteins support the conclusion that free zinc levels in prokaryotes are quite low (Ma et al., 2014; Moore and Helmann, 2005; Outten and O’Halloran, 2001).

Remarkably, these estimated levels of free zinc are approximately six orders of magnitude less than the total zinc concentration in these cells! So how does any organism accomplish this amazing feat? The key players in *B. subtilis* are summarized in Figure 1 (Moore and Helmann, 2005). Zinc uptake in zinc-deficient cells is mediated by the ZnuABC transporter complex. As free zinc levels rise in the cell, the Zur repressor protein is activated and it represses ZnuABC expression thereby limiting further zinc uptake. Zur also represses zinc-

independent paralogs of ribosomal proteins. The expression of zinc-independent L31 and L33 subunits in zinc-limited cells allows for the use of their zinc-binding paralogs as intracellular sources of zinc. The expression of a zinc-independent paralog of S14 allows for ribosome assembly to continue despite the paucity of zinc. Rising cytosolic free zinc also inactivates a second transcriptional regulator, the CzcA repressor whose function is to inhibit expression of the CadA and CzcD metal efflux proteins. Thus, in a zinc-overloaded cell, CadA and CzcD expression is high and the excess metal is exported outside of the cell.

Both Zur and CzcA are responsive to the pool of free zinc and its level is highly dependent on the zinc buffering systems of the cell. In the report by Ma et al., the authors present convincing data that bacillithiol (BSH) is a major component of that buffering system (Ma et al., 2014). BSH was first discovered as the major low molecular weight thiol in the bacterial phylum *Firmicutes* (Newton et al., 2009). This phylum includes *B. subtilis* and *Staphylococcus aureus* and is the most abundant phylum found in the mouse and human gut microbiota (Ley et al., 2006). BSH is the α -anomeric glycoside of cysteinyl-D-glucosamine with L-malic acid and is the functional alternative to glutathione (GSH) in these organisms (Figure 2). Like GSH, BSH acts in redox homeostasis as a thiol buffer system (Helbig et al., 2008; Helmann, 2011). What is novel in this new work is the implication of BSH in zinc homeostasis. The authors begin by showing that BSH binds zinc with an affinity consistent with its buffering role. BSH forms a $\text{Zn}(\text{BSH})_2$ complex with a cumulative binding constant β_2 of $\sim 10^{12} \text{ M}^{-2}$. This affinity would allow the physiological BSH pool of 1–5 mM to effectively buffer Zn in the pM–nM range. They further present evidence that BSH forms this complex using the cysteinyl thiolates and probably the malate carboxylates.

Using *Bacillus* mutant cells disrupted for BSH synthesis, Ma et al. go on to show that wild-type and mutant cells have similar levels of total zinc. However, when those cells are then treated with zinc, wild-type cells rapidly accumulate zinc into a low molecular weight pool while BSH mutants do not. BSH mutant cells also have reduced zinc tolerance when the major export systems are also mutated suggesting a role for BSH in zinc buffering. The impact of BSH zinc buffering was further supported by the observation that cells lacking BSH induce the zinc efflux systems at lower concentrations of extracellular zinc and this led to the conclusion that BSH mutants have higher levels of intracellular free zinc that are detectable by CzcA.

In addition, the authors found that thiol reactive agents such as Cd and diamide effectively increase expression of the CadA and CzcD efflux transporters by interfering with BSH buffering and releasing zinc to inactivate CzcA activity. Notably, higher levels of diamide could also induce these efflux systems by apparently attacking sites of protein-bound zinc (primarily the L31 and L33 ribosomal subunits) and cause zinc release from those sites. Finally, the authors show evidence that BSH may also donate zinc directly to some protein sites via ligand exchange reactions. Specifically, the presence of BSH accelerated the rate of zinc release by the CzcA protein in vitro and it seems likely that this process could be reversible as well. It is likely that a significant fraction of the metallation of zinc-binding proteins occurs via direct ligand exchange reactions with donor complexes such as $\text{Zn}(\text{BSH})_2$.

These results provide compelling evidence that BSH is a major zinc buffer in *B. subtilis* but it is unlikely to be the only buffering molecule. The identity of other buffering components is unknown. In addition, BSH synthesis is limited to some prokaryotes while the need for zinc buffering is universal to all organisms. This then raises the question of what molecules serve to buffer zinc in organisms that do not produce BSH? The more ubiquitous low molecular weight thiol, GSH, has been previously proposed to provide zinc buffering (Helbig et al., 2008). However, given its low affinity for zinc (binding constant $3 \times 10^4 \text{ M}^{-1}$), this seems unlikely (Krezel and Maret, 2006; Ma et al., 2014; Walsh and Ahner, 2013). Alternatively, many prokaryotes and eukaryotes express metallothioneins, small cysteine-rich metal binding proteins that provide a buffer for zinc in those cells (Babula et al., 2012; Robinson et al., 2001). Metallothionein's affinity for zinc and its abundance allows it to be an effective buffer in the pM-nM range (Krezel and Maret, 2006). Labile zinc has been observed in presynaptic boutons in mossy fiber neurons of the hippocampus and in secretory granules of pancreatic β cells, mast cells, intestinal paneth cells, and prostate epithelial cells (Domaille et al., 2008; Nicolson et al., 2009; Okada et al., 1983; Thompson et al., 2002). Ligands stabilizing these pools have not been defined but the high level of citrate in prostate granules and glutamate in mossy fiber presynaptic vesicles suggest that these molecules buffer Zn^{2+} in these compartments. Finally, some organisms, e.g. the yeast *Saccharomyces cerevisiae*, produce neither BSH nor zinc-binding metallothioneins. Organisms like this yeast may be the perfect place to look for other agents involved in buffering this critical nutrient. An intriguing candidate comes from the discovery of labile zinc forms in the mitochondrial matrix (Atkinson et al., 2010). A kinetically reactive cationic Zn^{2+} complex of low molecular mass was discovered in yeast and its level correlated with metallation of zinc proteins in the matrix. The ligand in this complex has yet to be identified.

Perhaps what is most exciting to me about this new research from Ma et al. is what the future now holds for this field of study. We now have an identified zinc buffering system in a genetically tractable organism with a well-characterized and multifaceted system of zinc homeostasis. This means that future studies can specifically address the function of zinc buffer systems and their influence on zinc protein metallation and other components of zinc homeostasis and tolerance. Given the evolutionary conservation of zinc buffering, these future studies will provide insights relevant to all organisms.

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Figure 1.

Components of zinc homeostasis in *Bacillus subtilis*. Zinc uptake is mediated by ZnuABC, a zinc transporter complex under the regulation of the Zur repressor. Zinc efflux is mediated by Cad and CzcD, which are under the control of the CzcA repressor. Both Zur and CzcA are regulated by the free zinc pool that is buffered by BSH. Notably, a significant fraction of the metallation of zinc-binding proteins may occur via direct ligand exchange reactions with donor complexes such as $\text{Zn}(\text{BSH})_2$.

Figure 2. Structure of BSH. BSH is the α -anameric glycoside of cysteinyl-D-glucosamine with L-malic acid. Figure courtesy of J. Helmann.