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GUEST COMMENTARY

A Galvanizing Story—Protein Stability and Zinc Homeostasis[▽]

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Zinc is an essential component of protein function in all living cells. The intracellular availability of zinc ions must be balanced against their potential toxicity. Maintaining optimal zinc ion levels requires the integration of zinc enzyme expression with zinc transport. Much of the effort to understand bacterial zinc homeostasis, as well as that of other metals, has focused on transcriptional regulation and the biochemical properties of the relevant transcriptional regulators. Less is known about the translational and posttranslational mechanisms of metal homeostasis in bacteria, although these are clearly important (9–11). The significance of protein stability and turnover is emphasized in the results of a study of the zinc-dependent ZntR transcriptional activator, which are presented in this issue (15). These new data provide a way to reconcile the results of previous biochemical and biological studies of ZntR function (6, 13, 18) and provide a better model for understanding zinc homeostasis and one which can be applied to other metals. This study underscores the importance of designing experiments that account for the dynamic nature of cell physiology and its effect on intracellular transition metal utilization.

There are many known zinc proteins in *Escherichia coli* (≥50), several of which are essential (e.g., RNA polymerase and tRNA synthetases). Growth conditions that change the level of zinc protein expression will require adjustments of intracellular zinc levels to ensure that sufficient metal is available for the generation of active proteins. Access to zinc will depend on the concentration of both extracellular zinc and that recovered from the turnover of other Zn-containing proteins. Zinc ions are transported into the cytoplasm via ZnuABC (14), although other import pathways exist (5). ZnuABC expression is regulated by Zur (14), a Zn-dependent transcriptional repressor homologous to the well-known Fur family of metal-dependent regulators (8). When Zur binds available intracellular zinc, it represses ZnuABC transcription, ultimately leading to cessation of zinc import via this pathway. Excess zinc ions are exported by the zinc-inducible exporter ZntA (1, 16), whose expression is regulated by ZntR (2), a member of the MerR family of transcriptional regulators (7).

TRANSCRIPTIONAL REGULATION BY Zur AND ZntR

O'Halloran and coworkers have demonstrated using in vitro transcription and metal-binding competition experiments that the *E. coli* Zur and ZntR proteins sense and respond to very low zinc concentrations ($<10^{-14}$ M [6, 13]). In vitro analysis of *znuA* and *zntA* transcriptional regulation showed a sequential response to available zinc; Zur-dependent repression of *znuA* (uptake) precedes ZntR-dependent activation of *zntA* expression (efflux). Measurements of steady-state intracellular zinc in *E. coli* suggested that the average concentration per cell is constant, regardless of growth medium, in the absence of zinc supplementation (~200 μM or ~200,000 atoms/cell [13]). It is important that this value is an average and the range of zinc levels observed from cell to cell remains unknown, nor was this value determined over a range of external zinc concentrations. Because the lowest level of zinc achievable in *E. coli* is approximately 10^{-9} M (1 molecule/fl), these observations suggested that zinc access to Zur and ZntR must be kinetically controlled to prevent constitutive transcriptional regulation by these two avid zinc-binding proteins. The resulting model for zinc homeostasis in *E. coli* invoked a series of unidentified ligand-exchange reactions between various zinc-binding factors (protein and small molecules) that would favor zinc enzyme assembly over zinc-dependent gene regulation (6). To date, no candidate for a zinc chaperone has been identified in *E. coli* or any other organism.

More recently, the transcriptional response of *znuA* and *zntA* expression to extracellular zinc has revealed that the dynamic nature of Zn-dependent transcriptional regulation by Zur and ZntR is not well described by the biochemically based model described above (18). Under steady-state growth conditions over a range of zinc concentrations, changes in *znuA* and *zntA* transcript levels are offset by a 1,000-fold difference in extracellular zinc concentration. A different picture emerges when the transcriptional response is monitored immediately following zinc stress. In this case, the ZntR- and Zur-dependent transcriptional responses after 5 min are coincident with respect to extracellular zinc concentration. Notably, after 30 min the ZntR-regulated *zntA* transcript level diminished significantly, suggesting that the cells adapt to high external zinc. Viewed independently, the steady-state and transient responses to zinc stress (chronic versus acute) provide different pictures of the biological function of Zur and ZntR, hinting at a more complex mechanism of control of Zn homeostasis than that provided by simple Zn-binding thermodynamics.

The results of these studies can be explained in part by the new study by Pruteanu et al. (15). Previously, this group dem-

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onstrated the role of the *E. coli* Clp proteases in controlling transcription factor levels under different growth conditions (4). Here, they examine the proteolytic stability of ZntR, which had been identified as a potential ClpXP substrate (12). ZntR, like other MerR family members, binds to DNA both in the absence and in the presence of ligand. ZntR activates transcription by changing the conformation of the promoter region in the presence of zinc ions. The half-life of ZntR is increased in the presence of zinc (from ~30 min to >60 min). By the use of site-directed mutants of ZntR defective in either zinc or DNA binding, this effect was shown to depend upon both the DNA- and zinc-binding capabilities of ZntR, although not equally so. Mutants defective in DNA binding showed a decreased half-life (~15 min), while mutants defective in Zn binding still had a ~30-min half-life, but it did not change in the presence of Zn. Thus, the stabilizing effect of Zn required a functional DNA-binding protein. It will be important to determine the ZntR concentration in the cell to understand whether the stabilizing effect of DNA binding depends on specific or nonspecific binding sites. If ZntR is in excess relative to the single copy of P_{zntA}, then the stabilization against degradation is achieved by a substoichiometric amount of specific DNA-binding site.

The Zn- and DNA-dependent stabilization of ZntR is likely due to the conformation change induced upon Zn binding, which probably occludes the structural determinants for proteolysis. ZntR appears to be intrinsically unstable to degradation. Efforts to crystallize full-length ZntR resulted in the structure of an N-terminally proteolyzed species (3). Curiously, crystallization required the presence of DNA, although DNA was not observed in the density map. This proteolytic susceptibility of ZntR may not be a common feature of the MerR protein family, as other members respond to molecules that are not required intracellularly and are therefore always toxic. Because zinc is essential for cell growth, premature activation of *zntA* expression might be counterproductive. As proposed by Pruteanu et al., the reduced half-life of ZntR ensures that *zntA* expression will occur only when intracellular zinc levels are high enough to produce a large-enough population of zinc-loaded ZntR. As a consequence, zinc efflux would not be induced by small changes in intracellular zinc that could be considered noise.

These results highlight the dynamic nature of intracellular metal physiology. More broadly, they emphasize the importance of designing experiments that explore a variety of timescales and utilize complementary experimental methods to properly understand metal ion homeostasis. In particular, many bacteria are more likely to face acute metal stress due to changes in extracellular growth environment that change metal availability or alter intracellular physiology. Studies of metal homeostasis at the single-cell level under steady-state conditions are now being reported (17). An understanding of the kinetics of metal transport and transcriptional regulation at the single-cell level will be key in understanding the dynamic processes of metal homeostasis. Monitoring metal ion transport at the single-cell level, although currently challenging technically, would reveal whether this is a continuous or discontinuous process. Similarly, the dynamics of intracellular metalloprotein turnover will release metal ions and the effect of this process on metal-dependent transcriptional regulation can be followed at the single-cell level. The results of such experiments will undoubtedly reveal that the concept of a fixed intracellular metal concentration is limited to a steady-state average for a population of cells and does not describe metal homeostasis in individual cells continually observed over a defined growth period.

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