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## Common Mechanisms of Bacterial Metal Homeostasis

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### Abstract

Transition metals are required for the function of nearly half the enzymatic machinery of organisms. This is particularly challenging for bacteria, which move through environments in which metal levels can vary by orders of magnitude. Exacerbating the situation is the fact that metals easily compete for enzyme-binding sites, with inappropriate metallation typically inhibiting enzyme function. Thus microbes work hard to acquire, balance, and sort their metal pools. This chapter surveys the common tactics by which bacteria control intracellular iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn). The focus is on *Escherichia coli*, for which enough information is available to attempt an integrated view. High-affinity import systems are regulated at the level of transcription by specific metal-sensing transcription factors; posttranslational controls have not yet been identified. If these importers are insufficient, then metal-sparing strategies are engaged for iron and zinc, the two metals that are needed to activate essential enzymes. At the other extreme, metal overload can result in chemical injuries (Fe, Zn, Cu) and the mismetallation of noncognate enzymes (Fe, Zn, Mn). Export systems are induced to avoid these outcomes. Underlying the entire situation is the question of whether metals are sorted among enzymes by thermodynamic affinities or whether chaperone systems override binding strengths. At present the author infers that Cu movement may sometimes be chaperone driven, but that the other metals reversibly sample protein binding sites and populate them according to the relative binding strengths of proteins and competing metabolite ligands. This conclusion emphasizes that metal pool sizes must be controlled and balanced.

### Introduction

A microbe can thrive in natural habitats only if it can correctly activate its numerous metalloenzymes. This process is difficult if metals are either under- or oversupplied by the environment. A variety of studies have indicated that mammalian hosts manipulate local metal availability to suppress the growth of invading microbes. Yet long ago bacteria evolved strategies to adapt to

fluctuations in metal availability. This review explores how metal deficiency or excess can interfere with enzyme activities, as well as the adaptive measures that bacteria adopt to cope with such stresses. These issues lay the groundwork for considering how this ploy by the host may or may not succeed in blocking microbial growth.

### The Root of the Problem

The challenges of metal homeostasis derive from the fact that contemporary life occurs under conditions that are strikingly different from those of the primordial world in which cellular biochemistry evolved (Anbar 2008; Dupont et al. 2010). That world was anoxic. Iron in its ferrous form was readily available, and its facility at both redox and surface chemistry triggered its recruitment into numerous enzymes. The biochemical capabilities that it provided became the foundations upon which metabolic pathways evolved. Those enzymes and pathways persist today.

Conversely, the anoxic world was one in which reduced sulfur species were stable and abundant. Consequently, soft metals—notably copper—were trapped in insoluble sulfide minerals. Early life forms apparently did not use this metal at all. About 2.8 Gya the newly evolved photosystem II began to generate molecular oxygen. Initially, oxygen was scavenged by reduced  $\text{Fe}^{2+}$  and sulfur species; it did not begin to accumulate until these were titrated from the seas, a billion years later. By then iron had become much more sparingly available and, conversely, copper was released as a soluble bioavailable species.

Contemporary organisms have thus inherited a requirement for iron that is not easy to satisfy. They feature a relative handful of Cu enzymes, which have apparently all arisen since the great oxygenation event (Ridge et al. 2008). Zinc utilization has also grown (Dupont et al. 2006). The expansion of Zn enzymes was once thought to have arisen from its scarcity in primordial sulfidic environments, but a recent analysis suggests that zinc might always have been bioavailable (Robbins et al. 2013). Indeed, unlike copper, zinc is widely used by, and is probably essential for, current-day anaerobes.

In contemporary environments, metal availability is inconsistent, particularly for organisms that transit between anoxic habitats, which retain the metal content of the ancient world, and oxic ones. It is thus perhaps fortuitous that studies of metal homeostasis began in *E. coli*, a facultative organism that makes its way in both worlds and which therefore must employ multiple strategies to satisfy its metal requirements.

### The General Case

This review will focus on metal usage by *E. coli*. Emphasis will be on the acquisition and control of iron, manganese, copper, and zinc. Subsequent sections

outline the ways in which these metals are employed, their routes of import, the hazards of overload, how regulatory proteins track their pool sizes, and the efflux and chaperone systems that cope with excess. Details vary among microbes; however, they follow a general pattern that can be laid out without getting lost in the details. A general discussion highlights the common challenges that organisms face in ensuring proper metallation of client proteins.

The fundamental problem is that although protein surfaces easily discriminate among organic substrates, they have trouble with metals. The divalent transition metals lack shape, have a common charge, and overlap considerably in the coordination geometries that they will tolerate. Nevertheless, they are rarely interchangeable in the enzymes that rely upon them—an indication that actionable differences exist. In redox enzymes these differences arise from the distinct reduction potentials of iron, manganese, and copper. In nonredox proteins the metals exhibit biases in their coordination geometries. Further, softer metals exhibit generally stronger ligand binding than do harder metals, in accordance with the Irving-Williams series: ligand affinities,  $\text{Mn(II)} < \text{Fe(II)} < \text{Co(II)} < \text{Ni(II)} < \text{Cu(II)} > \text{Zn(II)}$  (Irving and Williams 1948). Such differences cause variations in catalytic efficiencies when the metals are loaded into enzymes, and they provide the means by which importers, sensors, exporters, and enzymes can (imperfectly) discriminate among them.

### **How Does a Cell Provide the Correct Metal to a Divalent Metal-Binding Protein?**

This is perhaps the most fundamental problem in metals biology. In principle, two solutions exist. The first is that the protein has a strong kinetic or thermodynamic bias toward the correct metal. The second is that selection occurs at the level of a chaperone, which then guides the metal into a client protein. Both solutions occur.

Cytoplasmic chaperone systems have been verified for copper and nickel (Leitch et al. 2009; Farrugia et al. 2013) but not for the more widely used metals: iron, manganese, and zinc. Might their chaperone systems simply not have been discovered? Perhaps, but this is dubious. A protein-based chaperone typically requires a domain in the recipient protein to serve as its landing site (Schmidt et al. 2000; Padilla-Benavides et al. 2013). These have not been noticed in Fe-, Mn-, and Zn-using proteins, and in any case it would have been evolutionarily cumbersome for such domains to have arisen across a broad expanse of proteins that draw upon a common metal. (In a typical organism, only a few enzymes use copper and nickel, whereas scores may require iron, zinc, and manganese.) Further, the heterologous expression of Fe, Zn, or Mn enzymes from distant organisms is usually successful, which indicates that metal delivery persists. One would have expected that drift in protein–protein recognition would have degraded chaperone-based metal delivery to heterologous

proteins. In contrast, heterologous expression of Ni or Cu enzymes is problematic (Natvig et al. 1987).

If chaperones are not involved in delivering zinc or iron to their cognate proteins, then one infers that Zn enzymes tend to acquire zinc in preference to iron, and that Fe enzymes do the opposite, because these are the thermodynamically preferred outcomes in a given cell. At first this seems surprising, given that zinc tends to outbind iron in most proteins.

An example may help. ZntR is a transcription factor that responds to Zn overload. Its  $K_D$  for zinc in water is approximately  $10^{-15}$  M (Outten and O'Halloran 2001; Ma et al. 2011). It can quickly be deduced that Zn loading occurs by ligand exchange with other solutes, as opposed to the binding of zinc that is water coordinated (i.e.,  $Zn[H_2O]_n$ ), and this recognition is critical for thinking about how metals sample binding sites. The maximum second-order rate constant for a reaction in water is  $\sim 10^9$   $M^{-1} s^{-1}$ ; this rate constant means that every encounter between two reactants (say, zinc and a binding site) is productive (the zinc binds). If zinc were exclusively present at  $10^{-15}$  M  $Zn(H_2O)_n$ , then the binding rate would be the product of the rate constant and the concentration:  $10^{-6}$   $s^{-1}$ . The halftime for binding would be, at best, one week. The amount of aqueous zinc is inadequate to metallate this protein in a workable time frame.

The resolution is that zinc and presumably all metals move inside cells as exchangeable complexes with metabolites and with the surfaces of biomolecules. Thus the amount of zinc that is accessible to ZntR inside an overloaded *E. coli* cell is far higher than  $10^{-15}$  M and may approach the total Zn concentration ( $10^{-4}$  to  $10^{-3}$  M) (Outten and O'Halloran 2001). One might imagine complexes of zinc with glutathione or histidine that, for example, deposit the metal through ligand exchange reactions in the ZntR binding site.

Accordingly, the common statement that “there is no free zinc” (or copper or other metals) inside a cell should be understood as meaning that there is no fully hydrated metal. There are, however, substantial pools of metals that have not been stably incorporated into proteins, which are loosely bound by metabolites. The term “free metal” should really refer to this pool of unincorporated metal, although in frequent usage it has sometimes been confused with fully hydrated metal, which is vanishingly scarce and functionally irrelevant.

However, the metal dissociation constants of proteins, which by convention are measured against water, retain their utility. If ZntR is half-loaded with zinc, then the concentration of aqueous zinc is  $10^{-15}$  M but the concentration of a Zn-loaded metabolite might be  $10^{-6}$  M. If exchange reactions are quick, then loading times will be short enough such that ZntR can quickly sense a rise in the intracellular Zn pool. Returning to the problem of the thermodynamic distribution of metals, a Zn-dependent protein with a greater affinity for zinc than that of ZntR (say,  $K_D$  of  $10^{-17}$  M) would, in principle, be saturated with zinc at levels lower than those that occupy ZntR. In this way, ZntR would not

trigger the synthesis of Zn efflux systems unless Zn-requiring proteins are already activated.

Iron binds more poorly to proteins than does zinc, but it also binds more poorly to the cellular metabolites. Imagine an Fe-dependent protein with  $K_D$  values of  $10^{-15}$  M for zinc and  $10^{-8}$  M for iron, and a Zn-dependent protein with  $K_D$  values of  $10^{-18}$  M for zinc and  $10^{-7}$  M for iron. The intracellular concentrations of aqueous iron and zinc might be  $10^{-6}$  M and  $10^{-15}$  M, respectively (matching the  $K_D$  values of the metal regulatory proteins, ZntR and Fur; see below). If exchange reactions established thermodynamic equilibrium, the Fe-requiring protein would ultimately be 99% Fe bound and the Zn-requiring protein would be 99% Zn bound, even though both proteins inherently bind zinc better than iron. The metal occupancy would be determined by the balance between the relative availabilities and binding constants of the metals for a given protein.

This analysis depends on the notion that thermodynamic equilibrium is achieved. For this to occur, binding *in vivo* must be quick, and it must be reversible. Can spontaneous metal release into water be sufficiently fast to meet the second criterion, or must something pry out the metal? Consider the first case. For metal release by ZntR to water,  $K_D = k_{\text{off}}/k_{\text{on}} = 10^{-15}$  M. Since  $k_{\text{on}}$  cannot exceed  $10^9 \text{ M}^{-1} \text{ s}^{-1}$ , then  $k_{\text{off}}$  cannot exceed  $10^{-6} \text{ s}^{-1}$ . Again, this implies that spontaneous dissociation into water occurs with a half-time of one week—far too slow for the ZntR regulatory protein to succeed as a dynamic sensor of metal availability. We infer that for this protein to operate as a Zn sensor, the bound zinc must be transferred directly by ligand exchange to other metabolites. That is, metabolites grab zinc and pull it from the protein. A correct formulation of Zn binding by ZntR must be:



where L represents unknown nonaqueous ligands with substantial metal affinity.

This analysis indicates several things. First, for proper metallation, the pools of metals in the cell must be calibrated relative to one another so that each metal competes favorably for cognate proteins but unfavorably for non-cognate proteins. It must be the goal of regulatory proteins to set the pools in this way. Second, cellular metabolites must be available to coordinate metals and facilitate both metal binding to and dissociation from proteins. Third, the absolute concentrations of the metal pools inside cells must be high enough so that metalloprotein loading occurs in seconds, not hours.

In most cases we do not yet know the correct values for the intracellular metal levels and for the binding constants of metalloproteins. Presumably, they are indicated by the affinities of the regulatory proteins that tip the cell behavior toward further import or efflux. It is notable that the metal-binding sites of metalloregulators have evolved steric constraints which strongly favor cognate metals. CueR binds copper with high avidity; it does not bind zinc as

well (Changela et al. 2003). These preferences are primarily dictated by the geometries of the coordinating residues, and sometimes they are enhanced by allosteric effects which can be imparted by the cognate metals but not by mismatched metals (for an excellent review, see Ma et al. 2009). However, metalloenzymes have less flexibility to evolve these discriminatory features: their structures are constrained by the exigencies of substrate binding and catalysis. Hence, mismetallation is more likely.

Finally, it is important to note that a subset of metalloproteins almost certainly does not achieve binding equilibrium. Superoxide dismutases and proteins with structural Zn atoms, for example, bury their bound metals within the protein so that they are inaccessible to chelators. *In vitro* these metals are released only if the protein is partially denatured. It is unclear whether discrimination against noncognate metals occurs at the level of the association rate or whether noncognate metals fail to trigger protein folding and thus are vulnerable to extraction.

## Iron

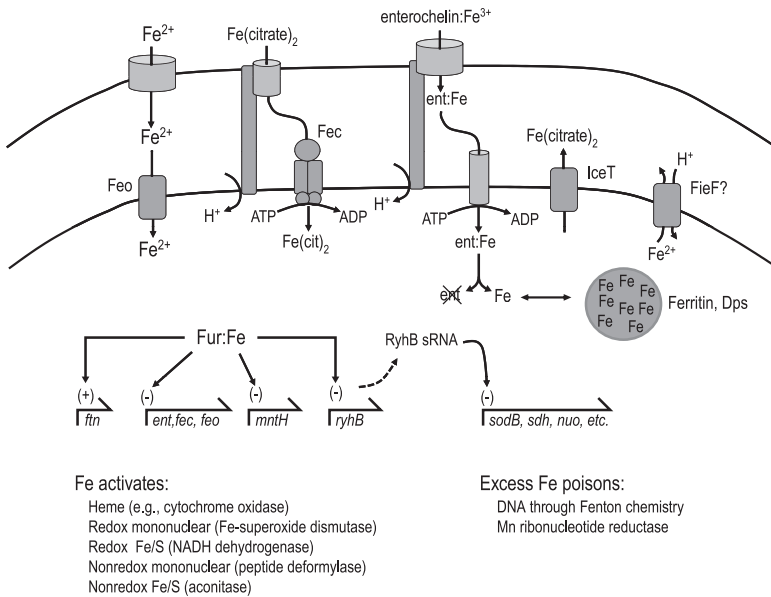
### *Chemical Properties and Enzymatic Roles*

Local ligands can tune the electron affinity of iron to a range of physiological potentials, making it a fitting partner in redox reactions. Iron also exhibits little activation energy when it switches between four-, five-, or six-coordinate geometries, a property which, together with its excellence as a Lewis acid, enables it to serve as a surface catalyst. In the most studied bacterium, *E. coli*, iron is by far the most-used transition metal. It serves in the form of heme as a conduit for electrons in cytochrome oxidases, succinate dehydrogenase, sulfite and nitrite reductases, and catalase. Iron-sulfur clusters in [4Fe-4S], [3Fe-4S], and [2Fe-2S] forms are redox-active cofactors in respiratory dehydrogenases (e.g., NADH dehydrogenase I), radical-generating enzymes (pyruvate:formate lyase-activating enzyme), and oxidant-sensing proteins (Fnr). Iron-sulfur clusters can also perform nonredox surface chemistry, binding and activating substrates for dehydration (aconitase); they also can apparently provide protein structure to many enzymes that operate upon nucleic acids (endonuclease III). Finally, lone Fe atoms provide redox-active mononuclear (superoxide dismutase) and binuclear (ribonucleotide reductase) centers, and in their ferrous form they provide substrate binding or activation sites in a wide variety of nonredox enzymes (erythrose-4-phosphate 3-epimerase).

These enzymes are found throughout metabolism: in energy production, amino acid and cofactor biosynthesis, nitrogen and sulfur assimilation, and DNA metabolism. Most bacteria cannot grow when they cannot acquire adequate iron.

*Avoiding Iron Deficiency*

It is an obvious problem that iron is hard to find in contemporary habitats. When iron is abundant, it is typically imported through ferrous Fe importers, such as Feo (Cartron et al. 2006) (Figure 5.1). When iron is scarce several ad hoc solutions come into play. In *E. coli* these tactics are initiated by the deactivation of Fur, an Fe-dependent transcription factor that in its Fe-activated form represses Fe-starvation responses (Hantke 2001). Fur binds a single ferrous Fe atom in a regulatory site with moderate affinity ( $K_D = 1 \mu\text{M}$ ; Mills and Marletta 2005; Ma et al. 2012), plus a Zn atom in a structural site with high affinity. A dimer of Fe-bound Fur [(Fur:Zn,Fe)<sub>2</sub>] binds DNA and occludes the RNA polymerase binding site of genes that are useful when iron is scarce, thus shutting them off. Fur:Fe displaces protein H-NS from upstream of the gene, encoding ferritin. Since H-NS would otherwise block *ftn* transcription, Fur:Fe displaces protein H-NS from upstream of the gene, encoding ferritin. Since H-NS would otherwise block *ftn* transcription,



**Figure 5.1** Iron homeostatic mechanisms in *E. coli* K12. Ferrous iron is imported through Feo, citrate-chelated iron through Fec, and siderophore-bound iron through the enterochelin system. The latter two systems depend on TonB to power entry through outer-membrane receptors. Siderophores are then hydrolyzed to release the iron. *E. coli* also expresses receptors (not shown) for siderophores excreted by other organisms. (Additional iron importers, including ones for heme, are found in pathogenic *E. coli* strains.) When iron is sufficient, moderate excess is stored in ferritin, bacterioferritin, or (during oxidative stress) Dps. Two iron export systems, IceT and FieF, have been proposed. Fur is the iron-activated repressor that controls expression of Fe importers, ferritin, the Mn importer MntH, and the sRNA RyhB. During Fe limitation, Mn may substitute for iron in mononuclear enzymes, and RyhB blocks the synthesis of expendable Fe proteins.



Fur:Fe has the effect of positively inducing it (Nandal et al. 2010). Thus, during periods of Fe repletion, the cell stores excess iron, to be drawn upon when Fe levels subsequently decline.

When intracellular iron becomes scarce, Fur is demetallated and thereby deactivated, allowing the engagement of adaptive responses. These responses include the induction of a variety of Fe import systems. Most famously, bacteria excrete siderophores (i.e., small molecules that solubilize ferric iron and deliver it to cells). The binding affinity of siderophores for Fe(III) is so great that after the import of the siderophore-iron chelate, the release of the ferric iron is an energetic problem. Release by ligand exchange to a moderate-affinity metabolite is energetically challenging, even if the latter is abundant. Release to a higher-affinity metabolite, such as citrate, would simply defer the problem. The native siderophore in *E. coli* lab strains is degraded by esterases (Brickman and McIntosh 1992). Lower-affinity siderophores apparently release the iron after its reduction by low-potential electron donors, a strategy that succeeds because the affinity of siderophores for ferrous iron is substantially lower than for the ferric form (Miethke et al. 2011; Wang et al. 2011b).

Pathogenic *E. coli* strains can also acquire their iron from heme, an important Fe source in their host environment (Torres and Payne 1997; Hagan and Mobley 2009). Lab strains, which were isolated from intestinal flora, cannot do this. Variation among the suite of Fe importers is commonly observed in otherwise similar bacteria, as lateral gene transfer is a device that suits bacteria to distinct habitats.

When siderophores are inadequate at acquiring sufficient iron, the cell adjusts further. *E. coli* does so in two ways. The derepression of the RyhB sRNA enables it to block the translation of messages that encode Fe enzymes that are abundant but conditionally dispensable (Masse et al. 2005). Repression of NADH dehydrogenase I, for example, spares the cell all the Fe atoms that would otherwise go into its nine iron-sulfur clusters, while NADH dehydrogenase II (an Fe-free enzyme) carries out the job of NADH reoxidation. The cell loses the proton motive force that NdhI contributes, but essential Fe enzymes (such as the cluster enzymes involved in isoprenoid synthesis; Loiseau et al. 2007) can still be activated. Similarly, succinate dehydrogenase (ten Fe atoms) is not made, prohibiting growth on TCA-cycle substrates like acetate, but growth on fermentable carbon sources remains possible. It is striking that glycolysis, the sole high-titer pathway that is not dispensable under any reasonable condition, contains no metal-dependent proteins.

Interestingly, some other bacteria prioritize the use of limited iron using control systems that are analogous, but not homologous, to RyhB. In *Corynebacteria*, for example, the protein RipA plays the role of RyhB sRNA: RipA is a repressor of Fe proteins and is itself repressed by DtxR when iron is sufficient (Wennerhold et al. 2005). This example underscores the general observation that metal control strategies are broadly distributed even though the specific mechanisms that execute those strategies may vary.



In *E. coli* the other key adjustment to Fe restriction is a shift toward the use of manganese rather than iron in key metalloproteins. MntH, the sole Mn importer, is repressed by Fur:Fe; when iron becomes scarce, the induction of MntH brings manganese into the cell (Kehres et al. 2002a). Manganese probably populates the nonredox mononuclear enzymes that would otherwise rely upon iron. These enzymes require a metal to neutralize the charge of an oxyanionic reaction intermediate, and manganese can do so almost as well as iron (Sobota and Imlay 2011; Anjem and Imlay 2012). Manganese cannot directly substitute for iron in the redox enzymes superoxide dismutase and ribonucleotide reductase, because the polypeptides of those Fe enzymes are configured to poise iron, not manganese, at the proper reduction potential for catalysis. Instead the cell synthesizes Mn-activatable isozymes: the Mn-specific superoxide dismutase and ribonucleotide reductase. Their structural genes (*sodB* and *nrdEF*) are repressed by Fur:Fe and thus are induced only when iron is scarce (Compan and Touati 1993; Martin and Imlay 2011).

One other adjustment to Fe depletion may also exist. *E. coli* contains two aconitases, each of which uses [4Fe-4S] clusters to catalyze their dehydration/rehydration reactions. Strikingly, and unlike clusters in other proteins, the cluster of aconitase B exists in equilibrium with the cellular pool of iron: when iron pools drop, the equilibrium shifts away from cluster stability, apoprotein accumulates and enzyme activity declines (Varghese et al. 2003). Activity does not fall to zero, but citrate levels automatically rise to a higher steady-state to push through the remaining enzyme. Some of the accumulated citrate is excreted. It seems plausible (though not proven) that the excreted citrate traps any available extracellular iron, thereby serving as a short-term emergency siderophore that reenters through the ferric citrate import system (Pressler et al. 1988). The connection between aconitase stability and Fe metabolism has also been seen in other organisms. In *Bacillus*, *Mycobacterium tuberculosis*, and mammals, apo-aconitase similarly accumulates upon Fe limitation and serves as an RNA-binding protein (Banerjee et al. 2007; Volz 2008; Pechter et al. 2013). The cleft vacated by the erstwhile [4Fe-4S] cluster binds conserved elements in mRNAs, stabilizing messages that encode Fe-import proteins and destabilizing those of Fe-storage proteins. There has been some hint that apo-aconitase might do the same in *E. coli* (Tang and Guest 1999).

Some lactic acid bacteria, which typically inhabit Fe-poor environments, have made the fateful commitment to eschew iron dependence (Archibald 1983; Posey and Gherardini 2000). They lack Fe-S and heme proteins, and thus they also lack the respiratory chain and TCA cycle that depend upon them. This compromise permits an independence from iron at the expense of less-efficient energy production. Interestingly, these bacteria commonly contain millimolar levels of manganese, suggesting that their mononuclear enzymes are likely populated by manganese in place of iron. These organisms employ Mn-dependent ribonucleotide reductase and superoxide dismutase. In essence, the Fe-deficiency adaptations that *E. coli* makes are only conditionally

constitutive in these other bacteria. As a final twist, these bacteria often employ pyruvate oxidases that release copious hydrogen peroxide into their environment (Pericone et al. 2000). Peroxide is a poison for the Fe enzymes of their competitors (Jang and Imlay 2007; Anjem and Imlay 2012), thereby providing an advantage for the lactic acid bacteria.

The take-home message is this: the central metabolic and biosynthetic pathways that are shared by all organisms evolved in an Fe-laden environment; contemporary organisms have thus inherited a dependence on a metal that is not reliably present. This situation has forced the evolution of ad hoc mechanisms for adaptation to Fe limitation. *E. coli*, an organism that routinely transits from an Fe-rich (anoxic) environment to a frequently Fe-poor oxic one, manifests these.

### *Control of Iron Levels: Other Aspects*

It is striking that manganese can bind in the regulatory metal-binding site of Fur (Mills and Marletta 2005; Ma et al. 2012). Fur:Mn can repress several members of the Fur regulon, although apparently not all of them. For example, Fur represses synthesis of the MntH Mn importer when either manganese or iron is abundant inside the cell (Kehres et al. 2002a; Ikeda et al. 2005). However, the mangano-superoxide dismutase is only repressed when iron is abundant; when iron is scarce (so that FeSOD cannot be activated) but manganese is abundant, MnSOD synthesis is robust (Pugh et al. 1984). These outcomes make physiological sense, but the physical mechanics of the protein that enable this selective effect are unknown.

IscR is an *E. coli* regulatory protein that controls the synthesis of the Fe-S cluster assembly machinery. It has a cluster binding site, and when [2Fe-2S] is loaded there, IscR substantially represses expression of the Isc assembly system (Schwartz et al. 2001). When cluster demand outstrips assembly, IscR shifts to its apoprotein form, enabling full synthesis of the Isc proteins. Interestingly, apo-IscR additionally acts as a positive transcription factor that induces expression of a secondary cluster assembly machine encoded by the *suf* operon (Yeo et al. 2006). The regulatory pattern makes sense, in that the Suf machinery requires iron levels that are lower than what is needed by the Isc machinery (Outten et al. 2004). The reason that Suf is less fastidious is not known.

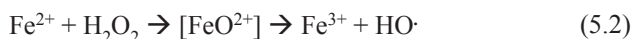
Recent studies have revealed that IscR also controls genes that have no obvious association with cluster assembly. These include NrdEF, a Mn-dependent ribonucleotide reductase (Cotruvo and Stubbe 2011; Martin and Imlay 2011). One possibility is that because IscR senses formation of a metal cluster, which can be built with iron but not manganese, it is a more precise sensor of Fe levels than is Fur, which responds to either iron or manganese. It remains to be seen whether this notion is correct.

Fur is widely distributed, but it is not the universal sensor of iron. For example, the Gram-positive bacterium *Bradyrhizobium japonicum* employs the

heme-sensing protein Irr as its primary transcriptional activator of Fe-import proteins. When adequate heme accumulates, Irr binds the heme and is converted it to a form that is rapidly degraded by cell proteases, thereby shutting down the synthesis of import systems (Yang et al. 2006). In an apparent analogy to IscR, this system results in specificity for iron. Interestingly, manganese has a secondary controlling effect: when Mn levels are high, manganese binds to Irr and stabilizes it (Puri et al. 2010). The effect is to maintain an Fe:Mn balance inside the cell. The consequence of an imbalance has not been determined.

### *Avoiding Too Much Intracellular Iron*

Too much iron is not a good thing. As will be demonstrated, excessive intracellular amounts of most metals are problematic because the excess metal outcompetes other metals for their appropriate binding sites. Iron does this in the limited case of Mn-specific redox enzymes (Whittaker 2003; Martin and Imlay 2011). However, the most recognized effect of excess iron is to drive the formation of hydroxyl radicals through the Fenton reaction:



Hydroxyl radicals react at nearly diffusion-limited rates with most biomolecules. The most consequential target is DNA (Imlay and Linn 1988). Since hydrogen peroxide is an unavoidable product of flavoenzyme autoxidation in oxic habitats (Imlay 2013), aerobic microbes are at pains to limit hydroxyl-radical formation by controlling the amounts of both reactants: loose ferrous Fe and  $\text{H}_2\text{O}_2$ . Deletions of *fur* that derepress Fe-importer synthesis in Fe-rich habitats lead to high rates of DNA damage (Touati et al. 1995). These can be suppressed by the engineered synthesis of ferritins, which lower the amount of free iron.

A complication to oxidative stress is that  $\text{H}_2\text{O}_2$  and superoxide both leach iron from dehydratase Fe-S clusters and mononuclear enzymes, increasing the pool of loose iron (Liochev and Fridovich 1994; Keyer et al. 1995; Anjem and Imlay 2012). Further, they knock iron free from Fur, with the potential effect of inducing Fe importers at the very time when more iron is a bad idea (Varghese et al. 2007). Cells respond in two ways. First, the OxyR transcription factor senses excess  $\text{H}_2\text{O}_2$  and induces higher rates of Fur synthesis, partially compensating for its lower metal occupancy (Zheng et al. 1999). Second, OxyR induces the expression of Dps (Altuvia et al. 1994), a ferritin-like protein that sequesters iron (Ilari et al. 2002). The effect is to down the level of free iron. Mutants that lack *dps* suffer overwhelming damage during periods of  $\text{H}_2\text{O}_2$  exposure (Park et al. 2005).

It is interesting to note that when Fe-starved strains were abruptly supplemented with iron, the intracellular Fe levels rose only modestly: from  $\sim 20 \mu\text{M}$  unincorporated iron to  $\sim 70 \mu\text{M}$  (Keyer and Imlay, unpublished data). Since the period of Fe starvation strongly induced import systems, what kept Fe levels

from sky-rocketing? One possibility is that influx was countervailed by an Fe efflux system. FieF (YiiP) has been proposed to act as a Zn or Fe efflux system (Grass et al. 2005b; Lu et al. 2009). In addition, Frawley et al. (2013) recently reported that MdtD (also known as IceT) acts as an Fe-citrate exporter, although the conditions of its expression are not yet clear. An intriguing alternative is that metal importers may be product inhibited. Control on the level of turnover has not yet been reported for most transition metal importers, but the bacterial MgtE magnesium importer is inhibited when cytoplasmic magnesium binds and induces a conformational shift that closes the pore (Hattori et al. 2009). To date the Zn or Fe efflux pump YiiP provides a rare example of a metal transporter whose turnover is clearly controlled by the allosteric action of its substrate metal (Lu et al. 2009). The fact that we do not know whether metals stimulate or inhibit most transporters comprises an important gap in our understanding of metal homeostatic mechanisms.

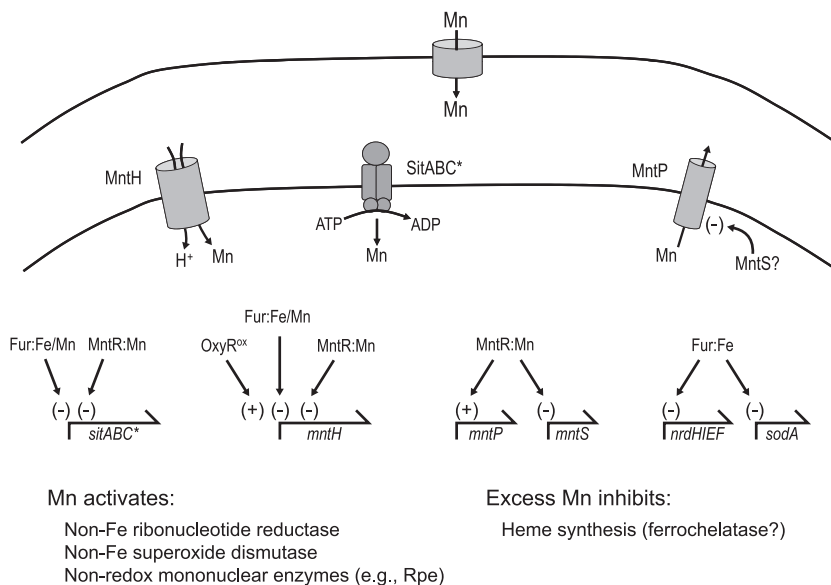
## Manganese

### *Chemical Properties and Enzymatic Roles*

Manganese is a transition element adjacent to iron in the periodic table. Like iron, it tolerates four-, five-, or six-coordinate geometries and can transition between Mn(II) and Mn(III) redox states at physiological potentials. These similar characteristics enable manganese to play many of the same roles as iron and, in fact the metabolism of these metals is intertwined (Figure 5.2).

*E. coli* expresses only a single Mn importer, MntH, a pump which is powered by the membrane potential (Kehres et al. 2000; Makui et al. 2000). Its close relative, *Salmonella typhimurium*, has an additional ATP-driven system: SitABC, also known in other bacteria as MntABC (Kehres et al. 2002b). Both the *sit* and *mntH* operons are repressed by an Fe-loaded Fur protein, with little expression under routine growth conditions (Kehres et al. 2002a; Ikeda et al. 2005). Indeed, the Mn content of *E. coli* is very low in nonstressed cells; for example, the Mn-dependent superoxide dismutase (MnSOD) is largely inactive (Anjem et al. 2009). Under these conditions the deletion of *mntH* does not cause any slowing of growth, even though intracellular Mn concentrations become miniscule. These observations suggest that manganese is specifically imported and employed in circumstances in which iron is unavailable. In fact, the deletion of *mntH* does block the growth of *E. coli* during Fe restriction (Grass et al. 2005a).

Regulatory studies revealed that *mntH* (of both *E. coli* and *Salmonella*) is also strongly induced during periods of hydrogen peroxide stress, as its transcription is positively controlled by the H<sub>2</sub>O<sub>2</sub>-sensing OxyR transcription factor (Kehres et al. 2002a). The *mntH* mutants cannot tolerate extended H<sub>2</sub>O<sub>2</sub> exposure (Anjem et al. 2009).



**Figure 5.2** Manganese homeostatic mechanisms in *E. coli*. Manganese is apparently used only during periods of Fe deficiency or  $H_2O_2$  stress. Upon deactivation of the Fur repressor or activation of the OxyR transcription factor, the MntH importer is synthesized and brings Mn into the cytoplasm. SitABC (MntABC) is found in many other bacteria, including the close relative *Salmonella*, but not in *E. coli* (hence the asterisk). Manganese levels are held in check at the transcriptional level by repression of *mntH* by both Mn-charged MntR and Mn-charged Fur. Excess Mn is exported by MntP; when Mn levels fall, MntP synthesis stops, and extant MntP may be inhibited by MntS. Two Mn-specific redox isozymes, Mn superoxide dismutase and Mn ribonucleotide reductase, are induced only during Fe restriction.

Analysis of the enzymes that can use manganese makes sense of these data. *E. coli* contains a number of enzymes in which iron serves as a nonredox cofactor. The cohort of such enzymes has not been adequately identified, because reducing conditions are needed to activate the enzymes with ferrous iron *in vitro*; for this reason some of these enzymes have been mistakenly annotated as using manganese, zinc, or cobalt. These enzymes (e.g., ribulose-phosphate epimerase, peptide deformylase, cytosine deaminase, LpxC) catalyze a variety of reaction types that have in common the formation of an oxyanion intermediate that is stabilized by electrostatic interaction with the divalent ferrous atom (Anjem and Imlay 2012). Lesser activity is exhibited when the enzymes are charged with zinc, which may be reluctant to assume the octahedral geometry of the enzyme:substrate complex, and essentially no activity is provided by magnesium. In contrast, manganese confers turnover numbers that approach those of the Fe-loaded enzymes. Thus the control of MntH synthesis by Fur makes sense if the imported Mn substitutes in these enzymes during Fe scarcity.

This hypothesis is supported by the fact that the structural genes for MnSOD as well as for the Mn-dependent ribonucleotide reductase, NrdEF, are similarly repressed by Fur:Fe (Compan and Touati 1993; Martin and Imlay 2011). These enzymes are induced only when iron is available; that is, when the usual Fe-dependent isozymes (FeSOD and NrdAB) cannot be activated. These are redox enzymes, and direct Mn substitution into the Fe-dependent enzymes cannot work, because the redox potential of manganese in those polypeptide environments is inappropriate for the reaction. Instead, the Mn isozymes are configured to poise manganese at the essential potentials. Thus the overall view is that Mn acquisition is a strategy to maintain the function of mononuclear and binuclear Fe enzymes when iron is unavailable.

The induction of MntH during H<sub>2</sub>O<sub>2</sub> stress is essential for continued growth; without it, the mononuclear nonredox enzymes are quickly inactivated by the oxidation of the ferrous cofactor by H<sub>2</sub>O<sub>2</sub> (Sobota and Imlay 2011; Anjem and Imlay 2012). In contrast, manganese does not react easily with H<sub>2</sub>O<sub>2</sub>, and so the Mn-loaded enzymes retain activity when exposed to H<sub>2</sub>O<sub>2</sub> *in vitro*. It has been inferred that this is the mechanism by which Mn import sustains the activities of these enzymes inside H<sub>2</sub>O<sub>2</sub>-stressed cells. However, direct evidence is lacking, because manganese binds weakly to active sites and dissociates during the process of enzyme recovery from cells. It is notable that lactic acid bacteria, which generate large amounts of H<sub>2</sub>O<sub>2</sub> via pyruvate oxidase, may be immune to this oxidant because they characteristically maintain millimolar intracellular pools of manganese (Archibald and Fridovich 1981; Daly et al. 2004). It appears that these bacteria have fully committed to the defensive strategy that enteric bacteria resort to only under stressful conditions.

This leads to an obvious question: If iron is often scarce and confers a vulnerability to oxidants, why don't the enterics routinely use manganese? One answer is that Mn-cofactored enzymes are not quite as efficient as Fe-cofactored ones (Anjem and Imlay 2012). Pursuant to the Irving-Williams series, manganese binds ligands more poorly than does iron; thus manganese binds enzymes less efficiently than iron, and in mononuclear enzymes it may also grip the substrate less effectively. A second point is that enteric bacteria would still require iron to populate heme and Fe-S clusters. Manganese cannot substitute. Since enterics must establish intracellular Fe pools in any case, the diversion of some iron to mononuclear enzymes is simple. Finally, enterics are facultative organisms which spend much of their lives in anoxic habitats. Iron tends to be reduced and available in that context. Thus the problems of oxidative stress and Fe scarcity might be relatively rare.

The last point raises the question of whether manganese might be more routinely used by bacteria that dwell in oxic habitats. This is probably the case. *B. japonicum* is an obligate aerobe which, in contrast to *E. coli*, grows very poorly without Mn import; defects arise from the low activities of MnSOD (its sole superoxide dismutase) and its Mn-dependent pyruvate kinase (Hohle and O'Brian 2012). This bacterium has committed to manganese as a cofactor.



Whether the selective pressure for this evolutionary decision arose from the frequency of metal availability or oxidative stress is unclear.

### *Avoiding Too Much Manganese*

Returning to *E. coli*, one can ask whether the cell sets upper limits on the amount of manganese that enters the cell. *E. coli*, like many bacteria (Rosch et al. 2009; Sun et al. 2010), has a LysE-family Mn efflux pump (MntP in *E. coli*) that pumps manganese out of the cytoplasm when levels rise too high (Waters et al. 2011). Indeed, *mntP* mutants are poisoned when a surfeit of manganese is provided in growth medium. Specifically, excess manganese poisons heme synthesis, apparently by blocking the insertion of iron into the porphyrin ring (Martin, Waters, and Imlay, unpublished data).

It is typical that bacteria encode both importers and exporters of the same metal. Not surprisingly, the syntheses of both are controlled by the cellular content of the metal. In the case of manganese, this situation is unexpectedly complicated: Mn levels are sensed both by MntR, a Mn-specific member of the DtxR family (Patzner and Hantke 2001; Galsfeld et al. 2003), and by Fur itself, which can form a Fur:Mn complex (Ma et al. 2012). MntR:Mn represses *mntH* and activates *mntP*: when intracellular Mn levels are high, the cell shuts down the synthesis of an importer and activates the synthesis of the exporter. When iron is scarce, repression of *mntH* by MntR:Mn is less effective. Upon MntH induction, the intracellular Mn levels rise quite high: ca. 100  $\mu\text{M}$ , compared to  $\sim 10 \mu\text{M}$  under routine conditions (Anjem et al. 2009). Thereafter Fur:Mn complexes form and repress the expression of *mntH* (Kehres et al. 2002a; Ikeda et al. 2005). This system might establish two different Mn set points: a low one when iron is available and a higher one when iron is scarce (or  $\text{H}_2\text{O}_2$  is present). A shift to high manganese in the latter situation makes sense if manganese must occupy mononuclear sites for which it has only moderate affinity. As an added wrinkle, when intracellular Mn levels are low, the synthesis of MntS (a sRNA that encodes a 42-amino-acid protein) is induced (Waters et al. 2011). MntS helps manganese find its way into Mn-using proteins, perhaps by inhibiting the activity of any extant MntP (Martin, Waters, Storz, and Imlay, submitted). When Mn levels rise, MntR:Mn inhibits *mntS* transcription; the effect may be to enable MntP to export the excess.

The ability of Fur to bind manganese in its Fe regulatory site has long been recognized (Ma et al. 2012). Fur:Mn is an effective repressor of many, but perhaps not all, genes in the Fur regulon. When manganese grossly overloads *mntP* mutants, Fur:Mn inhibits the synthesis of Fe importers, and heme synthesis stalls because the Fe levels become too low to sustain it.

In this context we can recognize why manganese is involved in controlling the Fe levels of *B. japonicum*. Irr is an inducer of Fe-import systems, but it is quickly degraded if it complexes with heme, an apparent indicator of Fe



sufficiency. Manganese can diminish the affinity of Irr for heme; this has the effect of stimulating greater Fe import if manganese is inside the cell (Puri et al. 2010). Since iron and manganese can problematically compete for some of the same proteins—including ferrochelatase, which synthesizes heme (Martin, Waters, Storz, and Imlay, submitted)—it seems possible that Irr is configured to avoid these conflicts by keeping the two metals in balance with one another. This scenario underscores that the optimal intracellular level of one metal is contingent on the levels of competing metals.

In summary, the regulation of Mn import by Fur, OxyR, and MntR in *E. coli* is configured so that Mn levels rise only when iron is scarce or H<sub>2</sub>O<sub>2</sub> threatens Fe-cofactored enzymes. The MntP efflux pump serves to remove manganese when its levels rise too high. Both the benefit and threat of intracellular manganese arises from its ability to occupy protein sites that normally acquire iron. In aerobes the evolutionary shift toward the use of manganese may have proceeded further. A key question is whether the Mn-using enzymes in these aerobes have refined their sites so that they are more effective with manganese than with iron.

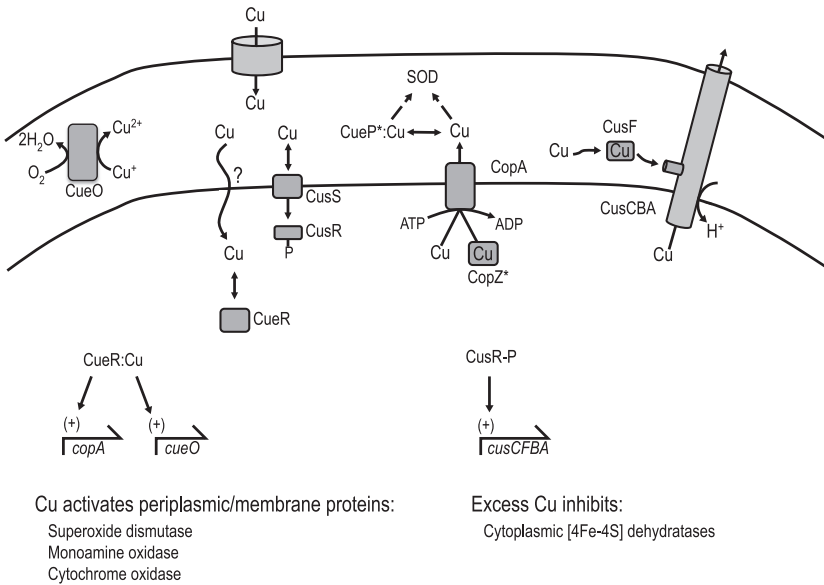
## Copper

### *Chemical Properties and Enzymatic Roles*

Copper has an unusual story. Because copper is a soft metal that binds tightly to sulfur ligands, in primordial environments it was trapped in sulfide precipitates (Anbar 2008; Ridge et al. 2008). Its unavailability meant that it was not employed as a cofactor in ancient microorganisms, and in contemporary organisms, it is used as a cofactor only in enzymes that have arisen since the accumulation of molecular oxygen, perhaps 2 Gyr ago.

Further, copper is not used in cytoplasmic enzymes—which, after all, inhabit a cellular compartment that resembles ancient Earth, with high concentrations of sulfur compounds that adhere tightly to cuprous copper. Instead, copper activates a relative handful of periplasmic and membrane enzymes that leverage its redox activity to perform electron-transfer and oxidation reactions. These include monoamine oxidases, superoxide dismutases, cytochrome oxidases, and plastocyanins (Arguello et al. 2013).

Thus bacteria strive to keep copper out of the cytoplasm and to maintain adequate levels in the periplasm (Figure 5.3). High levels of copper are toxic to bacteria, a trait that has recently been exploited in the manufacture of antimicrobial surfaces, such as hospital doorknobs and catheters. The sensitivity of bacteria to copper depends enormously on whether Cu-binding compounds are present in the environment: while 5  $\mu$ M copper can poison bacteria in minimal media, 5 mM may be necessary in LB medium (Macomber and Imlay 2009). Presumably the former is a better mimic of natural habitats.



**Figure 5.3** Copper homeostatic mechanisms in *E. coli*. The few (ca. 5) Cu-dependent enzymes of *E. coli* all receive their copper from the periplasm; there are no cytoplasmic copper enzymes. Copper can slip into the cytoplasm through unknown mechanisms. When it does, the CueR transcription factor detects elevated levels of cytoplasmic Cu and induces the synthesis of the CopA export system and the CueO periplasmic copper(I) oxidase. The latter may diminish Cu influx by eradicating the monocation. Although *E. coli* Cu enzymes apparently acquire Cu passively in the periplasm, excessively high periplasmic copper is sensed by the CusSR two-component system, which triggers synthesis of the Cus exporter. In some bacteria, periplasmic Cu-binding proteins (here, CueP) traffic Cu from the cytoplasmic Cu exporters to periplasmic Cu proteins.

### The Problem with Copper in the Cytoplasm

Toxic doses of copper block particular functions in *E. coli*. Copper exposure impedes the biosynthesis of branched-chain amino acids and the catabolism of TCA-cycle substrates because Cu(I) attacks the solvent-exposed Fe-S clusters of the dehydratases that lie in these pathways (Macomber and Imlay 2009). Copper apparently coordinates the bridging S ligands, thereby displacing iron and ultimately causing full cluster degradation. Workers have also postulated that the redox activity of copper might enable it to participate in Fenton-like reactions that generate toxic hydroxyl radicals, which can damage DNA. This chemistry occurs *in vitro* (Gunther et al. 1995). However, Cu-overloaded *E. coli* did not exhibit higher than normal levels of DNA damage, even when exogenous H<sub>2</sub>O<sub>2</sub> was supplied (Macomber et al. 2007). The likely explanation

is that cytoplasmic copper predominantly associates with protein and lipid surfaces, away from the DNA, so that its genotoxic effects were small relative to that of the Fe pool. Copper can also catalyze the oxidation of protein thiols, although to date this effect has not been demonstrated in live cells.

For most bacteria it is unclear how copper ever enters the cytoplasm. It seems plausible that Cu(II) might sneak in through metal transporters that intend to import different divalent metals, and that Cu(I) might ride in through monovalent (e.g., potassium) systems. In any case, bacteria universally maintain ATP-driven efflux pumps that efflux copper from cytoplasm to periplasm. These proteins are strongly induced during Cu exposure, and null mutants are noticeably copper sensitive. Copper toxicity and resistance has been examined in a number of organisms, with *E. coli* and *Enterococcus hirae* among those which might be regarded as paradigmatic. Synthesis of the *E. coli* exporter, CopA (Rensing et al. 2000), is induced when copper metallates CueR, a MerR-family transcription factor that in CueR:Cu form activates *copA* transcription directly (Outten et al. 2000). In some organisms (though not *E. coli*), CopA is apparently fed copper by small cytoplasmic Cu-binding proteins, which are thus regarded as chaperones (Gonzalez-Guerrero and Arguello 2008). Presumably they sweep copper off the surfaces of the myriad metabolites, proteins, and membranes to which it otherwise sticks. CueR:Cu also activates expression of *cueO*, a gene that encodes a periplasmic enzyme that oxidizes Cu(I) to Cu(II) with tetravalent reduction of molecular oxygen to water (Grass and Rensing 2001; Singh et al. 2004). CueO contributes substantially to Cu resistance. Most workers suspect that Cu(I) penetrates the cytoplasmic membrane more easily than Cu(II) does, so that this action of CueO lessens Cu influx. Unfortunately, the ability of copper to adhere to cell-surface molecules is so great that this superficial copper dwarfs cytoplasmic copper, even in *cueO copA* mutants, thereby precluding accurate measurements of the levels of cytoplasmic copper.

The measured  $K_D$  of CueR for copper is  $10^{-21}$  M (Changela et al. 2003). Its selectivity for Cu(I) arises from linear coordination by cysteine residues, a geometry that is incompatible with the principle divalent metals. This high affinity allows CueR to outcompete adventitious Cu ligands. Using the same argument presented earlier, we can conclude that there is no fully hydrated Cu in the cell; copper transits from one surface to another through ligand-exchange processes. Within the cytoplasm the most plausible solubilizers are thiols (cysteine, glutathione) and amines (histidine, polyamine). Presumably these or other Cu-binding molecules have enough affinity to extract copper from CueR, thereby deactivating it, when cytoplasmic Cu levels subside. The alternatives are that CueR:Cu is deactivated by Cu transfer to an unknown dedicated protein chaperone, which might in turn transfer it to CopA, or that CueR is degraded.

*Copper in the Periplasm: Sufficiency and Excess*

*E. coli* and many other bacteria manifest a second pump, CusCFBA, that expels copper from both the cytoplasm and the periplasm into the external environment (Franke et al. 2003). A small Cu-binding protein, CusF, binds excess periplasmic copper and feeds it into the CusCBA transmembrane export complex (Kittleson et al. 2006). Expression of these genes is activated by the two-component CusSR system; the sensor protein is localized in the cytoplasmic membrane and probably directly senses periplasmic Cu levels (Munson et al. 2000). Activation of CusSR requires higher levels of environmental copper than does CueR. Presumably the differences between these two responses provide a window of Cu concentration that enables effective activation of periplasmic enzymes without collateral overloading of the cytoplasm. It is also plausible that high levels of copper directly damage proteins or membranes exposed within the periplasm.

One of the most interesting, unresolved stories involves how copper finds its way to client proteins in the periplasm. Passive metallation is a possibility: superoxide dismutase apoprotein can be activated by simple incubation with copper (Krishnakumar et al. 2004). Indeed, if *Salmonella* is grown with scant copper, superoxide dismutase is synthesized and maintained in an inactive apoprotein form until copper is provided, at which point the enzyme immediately gains activity. It is striking that Cu enzymes, unlike Fe enzymes, are not regulated at the transcriptional level in response to the availability of their cognate metal.

However, in several bacteria it appears that CopA-type cytoplasmic efflux systems and periplasmic chaperones are important for efficient activation of both periplasmic superoxide dismutase and cytochrome *c* oxidase (Swem et al. 2005; Gonzalez-Guerrero et al. 2010; Osman et al. 2013). This implies that copper takes a circuitous route: from the extracellular environment to the periplasm, into the cytoplasm, and then through ATP-driven efflux pumps to periplasmic Cu-binding proteins, which ultimately insert the copper into their client apoproteins. If true, entry of copper into the cytoplasm is requisite for the activation of Cu enzymes. Still, no influx protein has been identified in most of these bacteria. *E. hirae* is an exception (Solioz and Stoyanov 2003).

Why would cells require Cu flow into the cytoplasm to metallate periplasmic proteins? If Cu efflux systems evolved before Cu-requiring proteins—a reasonable idea—then newly evolved periplasmic apoproteins may have found the efflux systems to be the most reliable single source of copper. Periplasmic Cu-binding chaperones may have originally served to sequester copper, after the manner of heavy-metal metallothionines, and subsequently evolved to pluck copper off the export systems and to deposit it onto client proteins. The latter would presumably have acquired chaperone docking sites to make the process maximally efficient.

Copper thus stands alone among metals in several ways that all trace back to the strong affinity with which it binds adventitious ligands. It activates only recently evolved enzymes, which are generally involved in oxygen-dependent processes; it is compartmentalized in the periplasm to avoid reactions between it and exposed Fe-S clusters; and it is trafficked by chaperones to minimize nonproductive associations with biomolecules.

## Zinc

### *Chemical Properties and Enzymatic Roles*

The Irving-Williams series indicates that zinc binds ligands with greater avidity than do most transition metals. Zinc is thiophilic, and initial calculations suggested that it was minimally available to the microbes that prevailed in anoxic, sulfur-rich primordial environments (Anbar 2008). A more recent analysis, however, suggests that zinc might not have been so scarce (Robbins et al. 2013). In any case, proteomics analyses indicate that zinc, unlike copper, was employed as an enzyme cofactor in ancient microbes, and it is employed by enzymes in contemporary anaerobes. Its severalfold greater use in eukarya (Dupont et al. 2006) might reflect higher bioavailability in oxic habitats, or it may merely reflect its expansive use in regulatory proteins.

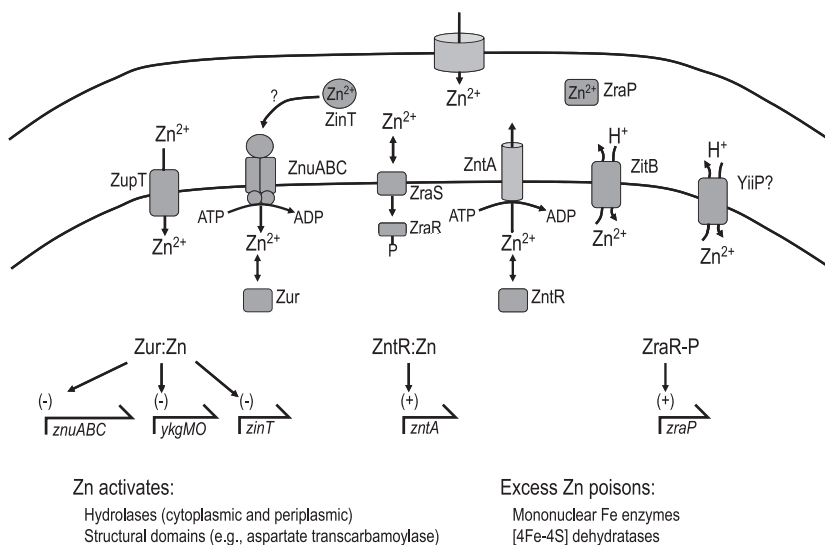
At physiological redox potentials zinc is present exclusively in the divalent Zn(II) state. The most common enzymatic use of zinc is as a Lewis acid that activates water as a nucleophile. In this role, zinc is present in many hydrolases, coordinated in its preferred tetrahedral geometry by three amino acids (e.g., His, Asp, Cys) and a water or hydroxide moiety (Vallee and Falchuk 1993). In some bacterial enzymes (e.g., aspartate transcarbamoylase; Helmstaedt et al. 2001) it plays a structural role. In the latter guise it is bound by four amino acids, often four cysteines, without any open coordination site. Higher organisms exploit zinc in Zn finger structures, wherein the avidity with which it binds His<sub>2</sub>Cys<sub>2</sub> coordination sites is so great as to organize the protein fold.

Zinc is distinguished from iron, manganese, and copper in that it populates enzymes both in the periplasm and the cytoplasm. One might rationalize the use of zinc in periplasmic proteins by the fact that, like copper, it is a metal that binds proteins tightly. In a compartment in which metals cannot be concentrated, this property might ensure full metallation. In contrast, iron and manganese bind relatively weakly to metal-binding sites, and so their local concentrations must remain high to ensure enzyme activity. Conversely, the thiophilicity of zinc does not match that of Cu(I), and it does not displace iron from Fe-S clusters as readily as does copper (Xu and Imlay 2012). Therefore, substantial levels of zinc can be tolerated in the cytoplasm.

## Ensuring Zinc Sufficiency

Zinc presumably enters the periplasm by passive movement through porins (Figure 5.4). Based on its binding behavior, one expects the environmental Zn ion to be associated with small organic compounds, and it likely enters biological Zn-binding sites through exchange reactions, without dissociation into a fully hydrated Zn(II) form. Entry into the cytoplasm is catalyzed by both ATP- and pmf-driven Zn importers. These are found throughout the microbial biota, usually with both types present in a single species. *E. coli*, for example, features both the ATP-driven ZnuABC system (Patzner and Hantke 1998) and the proton-driven ZupT importer (Grass et al. 2002). Typically, pmf-driven importers operate at higher turnover number but lower affinity; the converse is true of ATP-driven importers. ZupT is thus likely to be the predominant importer of zinc when zinc is relatively abundant, whereas the ZnuABC system is induced when cytoplasmic Zn levels fall (Wang et al. 2012a).

In *E. coli*, cytoplasmic Zn sufficiency is monitored by Zur, a Fur-family transcription factor with both structural and regulatory Zn-binding sites (Ma et al. 2011). Like other members of this family, the metallated [Zur:Zn]<sub>2</sub> form



**Figure 5.4** Zinc homeostatic mechanisms in *E. coli*. Zinc passively enters the periplasm through porins and is pumped into the cytoplasm by ZupT (possibly constitutively) and ZnuABC (during Zn scarcity). Scarcity also triggers the replacement of Zn-binding ribosomal subunits with Zn-independent ones (YkgM and O) as an apparent sparing mechanism. Excess cytoplasmic zinc is sensed by ZntR, which then induces the ZntA exporter to augment the constitutive ZitB exporter. YiiP/FieF may be an additional Zn (or Fe) exporter. Periplasmic zinc proteins apparently bind zinc directly. Excessive amounts of periplasmic zinc are sensed by the ZraSR two-component system, which induces ZraP, a periplasmic protein that may sequester zinc.

acts as a repressor of transcription. It is known to control only three *E. coli* promoters. It represses transcription of the structural genes for the ZnuABC importer, so that this high-affinity, energetically expensive system is induced only when Zn import through ZupT (or other permeases) is inadequate. A *znu* mutant is hypersensitive to Zn deficiency and accumulates less zinc (Patzner and Hantke 1998). This regulatory strategy resembles that for iron.

Zur:Zn also represses YkgM and YkgO (Graham et al. 2009; Hensley et al. 2012). These are Zn-free homologs of Zn-binding ribosomal subunits L31 and L36. During periods of Zn deficiency, the induction of these proteins apparently enables them to supplant the Zn-binding proteins; this action would diminish cellular Zn demand and may actually release “stored” zinc through the degradation of the extant Zn-loaded ribosomal subunits. The sparing effect might be substantial, given the high titer of ribosomal proteins inside the cell. This mechanism of Zn redistribution was first identified in *Bacillus subtilis* and *Streptomyces coelicolor* (Nanamiya et al. 2004; Owen et al. 2007; Shin et al. 2007).

The third Zur:Zn-repressed gene is *zinT*. ZinT is a 25 kDa protein that accumulates in the periplasm and exhibits tight ( $K_D \sim 20$  nM) Zn binding (Graham et al. 2009). Its role, however, is unclear. Although researchers have suggested that ZinT might serve as a Zn chaperone, perhaps capturing zinc and delivering it to the Znu importer, *zinT* mutants seem no more sensitive to Zn depletion than do wild-type cells.

These adjustments raise the underlying question: What is the most immediate physiological consequence of Zn deficiency? Whether or not zinc was involved in primordial microbes, in contemporary organisms it is apparently an essential metal. Zinc has been predicted to cofactor ca. 5% of *E. coli* proteins (Andreini et al. 2006b), including essential proteins ranging from RNA polymerase to cell wall amidases. True Zn deprivation is difficult to achieve because the metal taints lab glassware; however, exhaustive efforts by the Poole group have confirmed that Zn deficiency sharply diminishes growth (Graham et al. 2009). Their study did not identify the Zn-dependent enzymes that comprised the growth bottleneck, and it is not clear whether Zn-deficient organisms make metabolic compromises analogous to the RyhB-triggered abandonment of oxidative phosphorylation during Fe deficiency.

### *The Hazards of Excessive Zinc*

Zinc has long been recognized as an inhibitor of enzymes *in vitro*. Nanomolar concentrations inhibit a wide variety of enzymes through nonspecific binding to active site His and Cys residues. Perhaps a more avid mechanism of Zn toxicity *in vivo* is through competition with other divalent metals for metal binding sites: on transporters, regulatory proteins, and metal-dependent enzymes. Among transition metals, zinc is particularly problematic because its position on the Irving-Williams series ensures that it binds especially tightly to protein



ligands. The prediction then is that excess zinc will disrupt the function of enzymes that work best with other metals.

*E. coli* is strikingly resistant to zinc. This is testimony to the effectiveness of efflux systems, as sensitivity returns when they are deleted (Grass et al. 2001). When sufficient doses of zinc are added to poison *znt* mutants, at least two classes of enzymes are targeted. Zinc destroys the exposed Fe-S clusters of the same dehydratases that copper poisons (Xu and Imlay 2012). Presumably the mechanism is again that the softer metal binds either the coordinating cysteine residues or the bridging inorganic sulfide atoms of the cluster, displacing iron. Zinc is less effective at this effect than is Cu(I), silver, or mercury—again, in accordance with the greater thiophilicity of the latter metals. Manganese, a harder metal, has no effect on these clusters.

Zinc favors tetrahedral coordination environments, and this geometry is achieved by most Fe and Mn mononuclear enzymes. It is common for ferroproteins to acquire zinc during purification; once bound, the zinc does not easily dissociate. Therefore, it is not surprising that high levels of intracellular Zn poisons enzymes that have evolved to use ferrous iron as a cofactor metal. Ribulose-5-phosphate 3-epimerase (Rpe) is an example of such an enzyme (Gu and Imlay 2013). In its catalytic cycle its ferrous atom forms bidentate bonds to its substrate, activating it for reversible deprotonation; the product is then released. During this process the Fe atom shifts from four- to six-coordinate geometry and back. Iron is effective at this chemistry because of its ability to shift its coordination scheme, and manganese can effectively substitute. In contrast, while zinc binds, it furnishes < 5% the activity of iron. The reason is not clear: the role of the metal is to stabilize electrostatically the oxyanionic substrate intermediate, and all divalent metals should be able to do so. One possibility is that iron more easily shifts coordination geometries over the reaction course; another is that iron releases substrate ligands with more alacrity than does a softer metal like zinc. Yet zinc binds far more tightly to the protein: iron dissociates from Rpe with a halftime of a few minutes, while Zn dissociation is negligible in 8 hours. In fact, when *E. coli* is overloaded with zinc, Rpe activity declines, and the recovered enzyme is quantitatively occupied by zinc.

Interestingly, mismetallation of this type is exacerbated by oxidative stress (Gu and Imlay 2013). Both superoxide and hydrogen peroxide can oxidize the ferrous atoms of such enzymes, causing the ferric iron thus formed to dissociate. Remetallation is customarily rapid. However, when such stress is chronic, the repeated dissociation of iron allows zinc many opportunities to compete for the metal-free site, and eventually it wins. Rpe is probably just representative of several mononuclear nonredox enzymes that become mismetallated with zinc during periods of superoxide or peroxide stress. The same effect has been confirmed for 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, the Fe enzyme that initiates the aromatic biosynthetic pathway. Failure of this enzyme is likely the cause of the collapse of this pathway during oxidative stress (Sobota et al. 2014).

It is intriguing that although zinc binds extremely tightly to these enzymes *in vitro*, zinc is quickly removed from them *in vivo*: when oxidative stress is relieved inside cells, enzymes reacquire iron within 20 min (Gu and Imlay 2013). This observation suggests that something inside the cell helped extract zinc from these sites. Thiol compounds are the most obvious candidates, and indeed, cysteine (but not glutathione) replicated the effect *in vitro*. Cysteine is generally a better metal chelator than is glutathione, because cysteine provides both a thiolate and a nearby primary amine as metal ligands. In glutathione that amine is derivatized; thus glutathione binds metals poorly. The labilization of zinc by cysteine may be a model for how metals are constantly solubilized and guided away from low-affinity sites and toward high-affinity ones *in vivo*. Of course, it is possible that thiolate proteins (i.e., thioredoxins or glutaredoxins) can also serve this purpose. This has not yet been tested.

### *Avoiding Zinc Excess*

How does excess zinc enter a cell? The regulation of the *znuABC* genes by Zur offers compelling evidence that the Znu system serves the purpose of Zn transport (Patzer and Hantke 1998). The *znu* genes are not needed under routine growth conditions, indicating that other systems supply adequate zinc. One possibility is that import of zinc and other divalent metals is supported by general divalent importers. Metal importers powered by proton movement, rather than ATP hydrolysis, are generally less particular in their selection of metals, perhaps reflecting the thermodynamic price that must be paid to achieve substrate specificity. ZupT and MntH can each transport a variety of divalent metals, including manganese, cadmium, cobalt, and iron (Grass et al. 2005a; Taudte and Grass 2010). ZupT is generally regarded as a Zn importer: *zupT* overproduction accelerated the import of exogenous zinc, and *zupT* deficiency exacerbated the chelator-sensitivity of *znu* mutants (Grass et al. 2002). The PitA transporter imports divalent cations chelated by phosphate (van Veen et al. 1994); when exogenous Zn levels are high, deletion of *pitA* lessens the intracellular Zn load (Beard et al. 2000). In *Cupriavidus metallidurans*, the expression of both *zupT* and *pitA* was reduced when high levels of zinc were provided (Kirsten et al. 2011). Whether these systems evolved to be broad in their metal selection, or whether their apparent promiscuity is the outcome of extreme experimental conditions, is uncertain.

Nevertheless, it is clear that Zn overimport is a natural phenomenon, as bacteria universally express countermeasures to avoid it. ZntA is a P-type ATPase that exports zinc from cytoplasm to periplasm (Rensing et al. 1997). ZitB, a member of the cation diffusion facilitator family, does the same (Grass et al. 2001). Both are induced when *E. coli* is exposed to high levels of zinc. Careful measurements show that upon abrupt exposure to zinc, ZitB lessens the immediate Zn accumulation, while ZntA has a more pronounced effect at later points

in time (Wang et al. 2012a). The implication is that ZitB may comprise a basal export activity, while after-induction ZntA supersedes it.<sup>1</sup>

The induction of *zntA* is stimulated by Zn-bound ZntR (Brocklehurst et al. 1999). The responsiveness to zinc is sharpened by the fact that the Zn-free ZntR is subject to rapid proteolysis (Pruteanu et al. 2007). *In vitro* measurements indicate that the Zn dissociation constant of DNA-bound Zur is  $10^{-16}$  M, while that of ZntR is approximately fivefold higher; this suggests that the window between them represents the target Zn activity inside cells (Outten and O'Halloran 2001). Total Zn in *E. coli* is  $\sim 0.2$  mM. One infers that even exchangeable zinc is continuously coordinated by metabolites and the surfaces of biomolecules. As discussed above, aqueous metal provides a standard state for binding-constant measurements but is not regarded as physiologically relevant. By using a modified form of carbonic anhydrase as a Zn sensor, however, the Fierke group determined that the steady-state level of hydrated zinc inside wild-type *E. coli* is actually far higher, about 20 pM (Wang et al. 2012a). Since ZntR is not activated inside these cells—as judged from the fact that *zntA* and other ZntR-controlled genes are not induced—there is an apparent contradiction. One possibility is that the *in vitro* behaviors of either ZntR or the carbonic anhydrase construct did not represent their Zn affinity *in vivo*. Alternatively, Wang et al. considered whether activation might be kinetically determined; that is, that the activity of ZntR may depend on the speed with which it acquires zinc, rather than on its binding equilibrium per se. In principle, rapid activation might require Zn levels far in excess of femtomolar levels. However, they noted that during a post-pulse period of Zn efflux, the ZntR response shut down even while intracellular Zn levels were still in the low nanomolar range, as reported by the carbonic anhydrase construct. Absent additional regulatory wrinkles (or inadequate equilibration by the reporter protein), this result implies that the binding constant of ZntR determined *in vitro* does not apply *in vivo*. This conundrum is currently unresolved, but the work presents a creative experimental design to put questions about Zn homeostasis on a quantitative footing.

Finally, exposure to extracellular zinc activates a pair of two-component systems. First, the ZraS/ZraR system triggers synthesis of a single gene: *ZraP* (Appia-Ayme et al. 2012), which encodes a 15 kD Zn-binding periplasmic protein. Under high Zn conditions, ZraP is reported to bind 70% of the proteome-associated zinc (Sevenco et al. 2011). Although a chaperone role is possible, the function of ZraP may simply be to sequester or buffer zinc. ZraS differs from ZntR in that ZraS senses periplasmic Zn levels. Sequestration there, of course, could protect both periplasmic and cytoplasmic compartments from overloading. Second, the BaeSR system is also activated by exogenous zinc,

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<sup>1</sup> Complicating the picture: YjiP (FieF) is another CDF family member. It effluxes either Fe or Zn *in vitro* and is modestly induced by overload of either *in vivo* (Grass et al. 2005b; Lu et al. 2009). Its physiological substrate is uncertain.

and it stimulates the synthesis of proteins that also diminish cytoplasmic Zn levels (Wang and Fierke 2013). Two members of the regulon, MdtABC and MdtD, appear to be pumps, and the most obvious explanation is that they export zinc or zinc complexes. Why the cell would need so many efflux pumps is unclear.

## Summary

Metal metabolism is a major part of cellular biochemistry, and both metal deficits and excesses disrupt physiology. The particular challenge with metals is that they are similar enough that mistakes are made, but different enough that enzyme mismetallation impedes function. This review has attempted to summarize current knowledge of how metals are used, how their intracellular concentrations are controlled, and how cells cope with too much or too little. Perplexing questions remain:

- Are importers controlled at the level of enzyme activity, either by allostery or product inhibition? How about exporters?
- How are metals trafficked through the cell: by ligand exchange with a multitude of metabolites, or with a dedicated few?
- How are metals excised from inappropriate sites? Is the identity of a metal in a protein site determined primarily by binding thermodynamics or kinetics?
- Do optimal metal pool sizes depend on environmental circumstances? If so, how does the cell adjust its homeostatic mechanisms to change the pool size?
- How are metal regulatory proteins configured to resist or cope with metallation by noncognate metals?
- To what extent has evolution adjusted the metals used by particular enzymes to fit the metal availability of the local habitat? What is the nature(s) of these adjustments?

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**Edited by: Jerome O. Nriagu, Eric P. Skaar**

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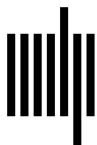
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