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Copper Chaperones: Personal Escorts for Metal Ions

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Copper serves as the essential cofactor for a number of enzymes involved in redox chemistry and virtually all organisms must accumulate trace levels of copper in order to survive. However, this metal can also be toxic and a number of effective methods for sequestering and detoxifying copper prevent the metal from freely circulating inside a cell. Copper metalloenzymes are therefore faced with the challenge of acquiring their precious metal cofactor in the absence of available copper. To overcome this dilemma, all eukaryotic organisms have evolved with a family of intracellular copper binding proteins that help reserve a bioavailable pool of copper for the metalloenzymes, escort the metal to appropriate targets, and directly transfer the copper ion. These proteins have been collectively called "copper chaperones." The identification of such molecules has been made possible through molecular genetic studies in the bakers' yeast *Saccharomyces cerevisiae.* In this review, we highlight the findings that led to a new paradigm of intracellular trafficking of copper involving the action of copper chaperones. In particular, emphasis will be placed on the ATX1 and CCS copper chaperones that act to deliver copper to the secretory pathway and to Cu/Zn superoxide dismutase in the cytosol, respectively.

KEY WORDS: copper; SOD; CCS; ATXI; ALS; metallocheperones.

THE REQUIREMENT FOR COPPER METALLOCHAPERONES

A diversity of enzymes are known to utilize copper as an essential cofactor (reviewed in Linder, 1991). At first glance, one might assume that such enzymes would have little trouble acquiring their cognate metal because copper typically accumulates in the cell at concentrations that far exceed typical binding affinities for the metal. Yet this naive assumption does not take into account availability of the metal. Copper is not only essential, but is also a potentially reactive and toxic ion, and accumulation of the free ionic form of copper is expected to be quite detrimental to the cell. As such, all organisms have devised cellular mechanisms for metal ion sequestration and detoxification that keep free ionic copper at extraordinarily low concentrations. In fact, we and others have shown that in spite of the micromolar quantities of copper that accumulate in a yeast cell, there is not a single atom that is freely available in the cytoplasm (Rae *et al.*, 1999). With this apparent "vacuum" of free copper, how can copper requiring enzymes acquire their metal? Fortunately, all eukaryotic cells have evolved with a specialized family of proteins that serve to spare copper for the enzymes that require the metal. These small copper binding molecules are globally known as "copper chaperones" (Pufahl *et al.*, 1997). Copper chaperones can acquire the metal under conditions where the metalloenzymes cannot, then function to deliver and directly transfer copper to specific cellular targets. In essence, these molecules act to escort copper ions and protect them from copper-scavenging detoxification mechanisms.

Thus far, three copper trafficking pathways have been identified that require the action of copper chaperones. These include: (i) copper delivery to the secretory pathway for activation of enzymes destined for the cell surface or extracellular milieu; (ii) copper trafficking to Cu/Zn superoxide dismutase (SOD1) in the cytoplasm; and (iii) delivery of copper to the mitochondria for activation of cytochrome oxidase. For the purposes of this review, we will

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focus on the two former pathways that involve molecules directly demonstrated to transfer copper to biological targets. These molecules are the ATX1 copper chaperone that delivers copper to the secretory pathway and the CCS copper chaperone for SOD1. In the case of copper trafficking to the mitochondria, a number of important candidates have been identified that cooperate in the insertion of copper into cytochrome oxidase (Glerum *et al.*, 1996a,b; Mattatall *et al.*, 2000), and the findings have been published in numerous reviews elsewhere and herein (e.g., Hamza and Gitlin, this issue; Harrison *et al.*, 2000; O'Halloran and Culotta, 2000; Valentine and Gralla, 1997). In any event, it is important to note that the players for all three copper trafficking pathways are well conserved throughout eukaryotes, yet were originally identified through genetic studies in the bakers' yeast *S. cerevisiae*. In this review, we will highlight the utility of exploiting yeast as a model system to identify and characterize copper chaperone molecules.

THE DISCOVERY OF THE PROTOTYPE COPPER CHAPERONE: YEAST ATX1

We originally identified yeast ATX1 as a putative antioxidant molecule (hence the name ATX) which when expressed to high levels in yeast, suppressed oxidative damage in cells lacking Cu/Zn SOD1 (Lin and Culotta, 1995). SOD1 is essential for scavenging toxic superoxide anions and yeast cells containing a *sod1*Δ null mutation exhibit a number of oxygen-dependent growth defects indicative of oxidative damage (Gralla and Valentine, 1991). The presence of *ATX1* on a multicopy plasmid was seen to suppress all these defects (Lin and Culotta, 1995). *S. cerevisiae ATX1* encodes a very small polypeptide of only 8.2 kDA and exhibits homology to a number of bacterial metal transporters, specifically the N-terminal metal binding domain of these transporters. As such, we hypothesized that ATX1 was capable of binding a certain metal ion and suppressed oxidative damage through a metal dependent mechanism. Indeed, copper ions were found to be required for the antioxidant function of ATX1 (Lin and Culotta, 1995).

How is copper-ATX1 capable of suppressing oxidative damage? Through a collaboration with Tom O'Halloran, a purified form of copper-ATX1 was seen to possess superoxide scavenging activity (Portnoy *et al.*, 1999). Although this reaction in vitro was not catalytic in the absence of an external reductant, we calculated that the activity obtained from a highly expressed ATX1 would be sufficient to substitute for SOD1 in yeast (Portnoy *et al.*, 1999). In spite of this antioxidant activity of overexpressed ATX1, the protein expressed at physiological levels does not appear to function as a true antioxidant. Instead, we found that this small copper protein acts in a novel fashion to shuttle copper ions within the cell.

The first indication that ATX1 was more than just an antioxidant was obtained through a collaboration with Andy Dancis (NIH). Specifically, a null mutation in *ATX1* was associated with loss of iron uptake (Lin *et al.*, 1997), a phenomena in yeast tightly coupled to copper trafficking (Dancis *et al.*, 1994). High affinity iron uptake in yeast requires a multicopper oxidase known as FET3 (Askwith *et al.*, 1994); this oxidase acquires its copper ions in the secretory pathway via a Golgi localized copper transporting ATPase. In yeast this transporter is known as CCC2 (Yuan *et al.*, 1995) and in humans, it is represented by the Menkes and Wilson Disease copper transporters which are affected in severe disorders of copper metabolism (see reviews in this issue by Lutsenko and coworkers, Fatemi and Sarkar, and Vorkoboinik and Camakaris). Through genetic epistasis and protein–protein interaction studies, we determined that CCC2 acquires its copper ions from ATX1 (Lin *et al.*, 1997; Pufahl *et al.*, 1997). Hence through a defined pathway, copper ions are sequentially passed from cytosolic ATX1 to the CCC2 copper transporter and then to FET3 in the secretory pathway (see Fig. 1).

The mechanism of copper transfer from ATX1 to the CCC2 copper transporter has been the subject of much investigation. First, it was noted that the copper transporter targets for ATX1 (e.g., yeast CCC2 or human Wilson and Menkes transporter) contain at their N-terminus, multiple copies of ATX1-like domains (Pufahl *et al.*, 1997). The sequence homology extends throughout the $\approx$8.0 kDa polypeptide, but most striking is a MTCXXC copper binding site that is unique to this family of protein domains. Through a collaboration with Tom O'Halloran and Jim Penner-Hahn, it was deduced that copper is transferred from the CXXC in ATX1 to the analogous site on the transporter via an interconversion between 2 and 3 coordinant copper-thiol binding intermediates (Pufahl *et al.*, 1997). This represented the first documented mechanism of copper transfer and ATX1 was denoted as the prototype "copper chaperone" or "metallochaperone" (Pufahl *et al.*, 1997).

How does ATX1 dock with its copper transporter target? By yeast two hybrid approaches, we demonstrated a copper-dependent physical interaction between ATX1 and the N-terminal metal binding domains on CCC2 (Pufahl *et al.*, 1997). As expected, the copper binding cysteines of the MXCXXC copper binding site were required for this interaction. In addition, a basic face of the ATX1 polypeptide was found to enhance interaction (Portnoy *et al.*, 1999). It is noteworthy that the analogous surface on the ATX1-like domains of the transporter is acidic

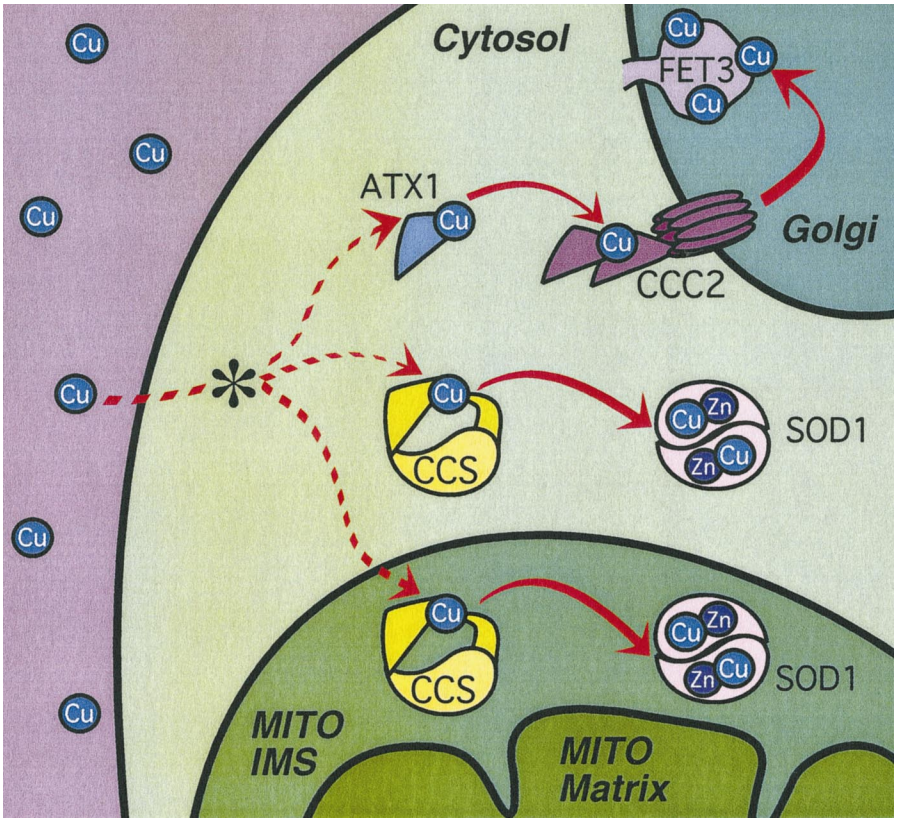

Fig. 1. Depicted is a cartoon illustrating two copper trafficking pathways in yeast involving the ATX1 and CCS copper chaperones. ATX1 (*blue triangle*) targets the metal to the Golgi P-type ATPase CCC2. Two tandem ATX1-like domains (*purple triangles*) at the N-terminus of CCC2 are the target of copper delivery by ATX1; the metal is ultimately delivered into the Golgi lumen leading to metallation of the multicopper oxidase FET3. The copper chaperone for SOD1, CCS, is responsible for inserting copper into Cu/Zn-SOD1. The three structurally distinct domains of CCS play separate roles in the copper transfer mechanism as described in text. CCS also controls the partitioning of SOD1 between the cytosol and the intermembrane space of mitochondria (*MITO IMS*). The mechanism by which the copper chaperones obtain the metal is not known, but does not appear to involve a direct hand off from cell surface transporters. Apparently an upstream "middle person" (indicated by an asterisk) may relay the metal between the transporters and the specific copper chaperones downstream.

rather than basic. We therefore proposed that docking involves ionic interactions between the negatively and positively charged surfaces on ATX1 and the copper transporter (Portnoy *et al.*, 1999). This model for docking has since been substantiated through elegant structural studies by Rosenzweig, Banci and O'Halloran (Arnesano *et al.*, 2001; Banci *et al.*, 2001; Rosenzweig *et al.*, 1999).

The bakers' yeast *S. cerevisiae* is of course not the only organism to express ATX1 and Jonathan Gitlin was the first to clone and describe the human homologue, denoted ATOX1 or HAH1 (Klomp *et al.*, 1997). Dr. Gitlin also succeeded in generating a knock out mouse model for ATOX1 and interesting, the mice are associated with a low survival rate and exhibit severe symptoms of perinatal copper deficiency (Hamza *et al.*, 2001). We find that human ATOX1 expressed in yeast nicely complements an *atx1*Δ mutation and can efficiently deliver copper to the *S. cerevisiae* copper transporting ATPase (Klomp *et al.*, 1997). The strong conservation of this copper trafficking pathway underscores the importance of carefully controlling delivery of copper to enzymes in the secretory pathway.

IDENTIFICATION OF THE CCS COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE

Superoxide dismutase 1 (SOD1) is an enzyme that is largely cytosolic and employs a copper cofactor

to catalytically disproportionate two superoxide anion molecules to hydrogen peroxide and oxygen (McCord and Fridovich, 1969). We found that in living cells, SOD1 relies on a copper chaperone to acquire its essential copper cofactor. The ATX1 copper chaperone described above plays no role in activating SOD1. Instead SOD1 acquires its copper through the action of a larger copper binding protein we have termed CCS, for copper chaperone for SOD1.

We first identified CCS as the product of the *S. cerevisiae LYS7* gene (Culotta *et al.*, 1997). *LYS7* was so named in the late 1960s based on the lysine auxotrophy (inability to grow on medium lacking lysine) of *lys7* mutants (Broquist, 1971). However, LYS7 is not directly needed for lysine biosynthesis, but instead functions to activate SOD1 with copper which in turn protects the lysine biosynthesis pathway from oxidative damage (Culotta *et al.*, 1997; Gamonet and Lauquin, 1998).

As initial evidence for a copper chaperone function for *LYS7*, yeast*lys7* null mutants were seen to be devoid of SOD1 activity in spite of accumulating normal levels of the SOD1 polypeptide (Culotta *et al.*, 1997). Through work with Jonathan Gitlin, SOD1 was demonstrated to be apo for copper and incapable of acquiring radiolabeled ^{64}Cu in cells lacking LYS7 (Culotta *et al.*, 1997). We identified the human homologue (also denoted as CCS) through a database search. A mouse knock out model for CCS has been created by P. Wong and J. Gitlin and most of the SOD1 polypeptide in this mouse appears apo for copper (Wong *et al.*, 2000). When the human CCS is expressed in yeast, this copper chaperone readily delivers copper to fungal SOD1 (Culotta *et al.*, 1997). As with the ATX1 pathway, the CCS copper transfer process appears nicely conserved between yeast and humans.

The discovery of the CCS copper chaperone prompted numerous investigations addressing the copper transfer mechanism of this curious copper chaperone. Such investigations included various spectroscopic, biochemical and structural studies. The findings have been published in numerous papers and reviews (Eisses *et al.*, 2000; Hall *et al.*, 2000; Huffman and O'Halloran, 2001; Lamb *et al.*, 1999, 2000a,b, 2001; Lyons *et al.*, 1998; Rae *et al.*, in press; Torres *et al.*, 2001; Zhu *et al.*, 2000), yet for the purposes of this report, we will focus on the molecular genetic studies in yeast that seeded the current thinking of how copper moves from CCS to SOD1.

By analysis of protein sequence alone, a complex mechanism of copper transfer is anticipated for CCS. Unlike ATX1 which consists of a single protein domain (Rosenzweig *et al.*, 1999), CCS is comprised of three structurally and distinct domains that carry out the separable functions of copper binding, copper transfer and docking with the SOD1 target (Fig. 1).

At the amino terminus, CCS domain I exhibits striking homology to ATX1, including the MXCXXC copper binding site, and crystallographic studies by Rosenzweig and colleagues have confirmed the structural conservation of this domain to ATX1 (Lamb *et al.*, 1999). Although this domain was suspected to be essential for copper transfer to SOD1, we found that a deletion of this domain only crippled CCS function and that copper transfer in vivo was still possible as long as yeast strains were not starved for copper (Schmidt *et al.*, 1999a). As such, we proposed that CCS domain I only facilitates copper binding under conditions of copper starvation, but is not the primary donor of copper for SOD1 (Schmidt *et al.*, 1999a).

The central domain of CCS (domain II) is the largest, $\approx$16.0 kDa. This domain bears striking homology to the target of CCS, the SOD1 enzyme (Casareno *et al.*, 1998; Lyons *et al.*, 1998; Schmidt *et al.*, 1999a). In fact, in the case of human CCS, the sequence identity between domain II and human SOD1 is close to 50%, with all the zinc binding ligands preserved and 3 of the 4 copper binding histidines of SOD1 present in human CCS. The fourth histidine is an aspartic acid at amino acid position 200 in the case of CCS (Schmidt *et al.*, 1999b). In spite of this strong sequence conservation to SOD1, we found that CCS possesses no SOD activity (Schmidt *et al.*, 1999b). However, we succeeded in turning CCS into a SOD simply by substituting the aspartic acid at position 200 with a histidine. In fact, this altered CCS molecule could function as both a copper chaperone and a SOD1 and appeared to activate itself with copper (Schmidt *et al.*, 1999b). This raised the question: why do eukaryotes go to the trouble of expressing separate molecules for the SOD1 copper chaperone and the SOD1 enzyme? As one possibility, a separate copper chaperone might allow for tight control of SOD1 activity. SOD1 in general is a highly expressed protein in virtually all cells and tissue types, however tissue variations have been noted in degrees of copper loading to the enzyme (Petrovic *et al.*, 1996; Rossi *et al.*, 1994, 1997; Steinkuhler *et al.*, 1994). As CCS generates active SOD1 protein through posttranslational metallation of apo-SOD1, SOD1 activity can quickly respond to oxidative stress without the need to synthesize the protein de novo.

The strong homology of CCS domain II to SOD1 serves to facilitate docking between the copper chaperone and its target. SOD1 is normally a homodimer and it was proposed that formation of a heterodimer between CCS and SOD1 may be a prerequisite to copper transfer (Casareno *et al.*, 1998; Lamb *et al.*, 1999; 2000a,b; Schmidt *et al.*, 1999a). In accordance with this model, domain II and SOD1 efficiently interact by two hybrid

and this interaction requires residues at the predicted dimer interface (Schmidt *et al.*, in press). Most striking, Rosenzweig and collegues have recently obtained cocrystals of yeast CCS and SOD1 and indeed these two molecules form a heterodimer that nicely parallels the SOD1 homodimer (Lamb *et al.*, 2001). While CCS domain II is necessary for interaction with the SOD1 target, this is not the domain that transfers the metal. Our studies indicate that this function is provided by domain III at the C-terminus.

Domain III is the smallest of the CCS domains, only 30 amino acids in the case of yeast SOD1. However, we noted that this is the most conserved region across CCS from diverse species and this sequence harbors an invariant CXC motif that is capable of binding copper (Schmidt *et al.*, 1999a). A single mutation in either of these cysteines is sufficient to abrogate copper transfer to SOD1 (Schmidt *et al.*, 1999a), but does not appear to inhibit docking with SOD1, as determined by two hybrid analyses (Schmidt *et al.*, 2000).

Taking all these findings into account, we have developed a model for how CCS transfers copper to SOD1 (Schmidt *et al.*, 1999a). The N-terminal ATX1 like domain is essential for capturing and binding copper, particularly under copper limiting conditions, and may interact with the C-terminal CXC copper binding site. Domain II in the central portion does not appear to bind or transfer copper, but is rather involved in docking with the SOD1 target. Finally, the ultimate step of copper transfer is accomplished by the small domain III at the C-terminus. This simple model built on molecular genetic studies in yeast have been substantiated and expanded upon by spectroscopic, biochemical and crystallographic studies in the laboratories of Rosenzweig, Valentine, Blackburn, and O'Halloran (Eisses *et al.*, 2000; Hall *et al.*, 2000; Lamb *et al.*, 1999, 2000a,b, 2001; Lyons *et al.*, 1998; Rae *et al.*, 2001; Zhu *et al.*, 2000).

A ROLE FOR CCS IN CONTROLLING THE CELLULAR LOCALIZATION OF SOD1

Historically, SOD1 has been characterized as a cytosolic enzyme. Most eukaryotes express a second distinct SOD enzyme that utilizes manganese as a cofactor and is localized to the matrix of the mitochondria. A mitochondrial form of SOD makes good sense in that the bulk of cellular superoxide is generated as a byproduct of electron transport in the inner membrane of mitochondria. We and others have recently found that in addition to the mitochondrial matrix, the intermembrane space (IMS) of mitochondria also possesses superoxide scavenging activity and this can be ascribed to a fraction of Cu/Zn SOD1 that enters the mitochondria (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001).

The level of SOD1 that accumulates in mitochondria represents a small fraction of the total ($\approx$1–2%). However, when one considers the predicted volume of the mitochondrial IMS compared to the cytoplasm, the concentration of mitochondrial SOD1 is predicted to be at least as high as cytoplasmic SOD1 (Okado-Matsumoto and Fridovich, 2001; Sturtz *et al.*, 2001).

As would be expected, a fraction of CCS colocalizes with SOD1 in the IMS of mitochondria where it is needed to specifically activate mitochondrial SOD1 with copper (Sturtz *et al.*, 2001) (Fig. 1). The presence of active SOD1 in the mitochondrial IMS presumably serves to scavenge the superoxide generated on the IMS side of the inner membrane. In fact, we have shown that mitochondrial SOD1 plays a physiological role in guarding against mitochondrial oxidative damage and helps extend the life span of aerobically grown yeast cells (Sturtz *et al.*, 2001).

How do CCS and SOD1 enter mitochondria? Neither contain a cleavable N-terminal presequence that is characteristic of many mitochondrial proteins; however, not all IMS proteins contain such presequences, e.g., cytochrome *c* (Nye and Scarpulla, 1990). In the case of SOD1, it is clear that mitochondrial uptake of SOD1 is strongly influenced by CCS.

The level of SOD1 that enters the mitochondria is directly proportional to the level of mitochondrial CCS. We found that in cells expressing CCS predominantly in the cytosol, mitochondrial levels of SOD1 are very low and conversely, targeting the bulk of CCS to the mitochondrial IMS results in a striking increase in mitochondrial SOD1 (Sturtz *et al.*, 2001). It would almost appear that "SOD1 follows CCS" into the mitochondria. Although the precise mechanism by which CCS controls SOD1 localization is not yet clear, studies indicate that both protein–protein interaction and metallation of SOD1 are involved (Field and Culotta, in preparation).

These studies on the mitochondrial form of SOD1 and CCS may be of particular relevance to a devastating and fatal motor neuron disease in humans known as amyotrophic lateral sclerosis (ALS) or more commonly, Lou Gehrig's disease. A subset of inherited cases of ALS are due to dominant gain-of-function mutations in SOD1 (Deng *et al.*, 1993; Gurney *et al.*, 1994). Although the mechanism of toxicity by mutant SOD1 is still unresolved (Cleveland and Liu, 2000), evidence points to a possible role of mitochondria in the disease (Beal, 2000; Borthwick *et al.*, 1999; Canto and Gurney, 1994; Dhaliwal and Grewal, 2000; Higgins *et al.*, 2002; Okado-Matsumoto and Fridovich, 2002; Wong *et al.*, 1995). As such,

controlling the mitochondrial import of SOD1 via CCS may provide new tools for addressing the role of mitochondrial SOD1 in familial ALS.

WHERE IS THE COPPER COMING FROM?

The method by which the copper chaperones acquire their metal ion is still not known. As one possibility, the molecules may go directly to "the front door" to obtain the metal from cell surface copper transporters. However, all efforts thus far to demonstrate protein–protein interactions between the copper chaperone and cell surface transporters have failed (Pena *et al.*, 2000; Portnoy *et al.*, 2001). As an alternative approach, we have used yeast genetics to survey a wide range of copper transporters for their ability to donate copper to ATX1 and CCS (Portnoy *et al.*, 2001). These transporters include the CTR1 and CTR3 high affinity copper transporters at the cell surface (Dancis *et al.*, 1994; Knight *et al.*, 1996), the FET4 low affinity divalent metal transporter (Dix *et al.*, 1994), and CTR2 (Kampfenkel *et al.*, 1995), a copper transporter localized to the vacuolar membrane in yeast (Portnoy *et al.*, 2001). The surprising result is that all these transporters can contribute to copper delivery to ATX1 and CCS. The copper chaperones exhibit no preference for a particular transporter (Portnoy *et al.*, 2001). These four distinct copper transporters exhibit no obvious sequence homology, making it difficult to envision a model in which the copper chaperones specifically dock with each of these molecules (Portnoy *et al.*, 2001). Instead it would appear that a "middle person" exists to ferry the copper between the transporters and the copper chaperones (Fig. 1 asterisk). Although the identity of this factor is not known, the utility of yeast genetics may again prove effective in revealing the nature of elusive copper trafficking factors.

PERSPECTIVES

Since copper is essentially a toxic nutrient, the trafficking of this metal inside cells must be carefully controlled to avoid unwanted adventitious copper chemistry. In part, this regimented trafficking involves the action of copper chaperones such as ATX1 and CCS. Although analogous molecules for other heavy metals have not yet been identified, such players are likely to exist. As with copper, redox active metals such as iron are dangerously reactive towards biomolecules and are not likely to randomly diffuse in the free ionic form. Even zinc, which is considered to be a relatively nontoxic metal can cause havoc to cells and recent studies by Outten and O'Halloran have shown that in bacterial cells, there is no free zinc (Outten and O'Halloran, 2001). With the apparent absence of freely circulating metal ions, metalloenzymes of all types face the common challenge of finding their cognate cofactor. Therefore, we anticipate that in the coming years, new "metallochaperones" will emerge that act in the intracellular trafficking of other heavy metals that are toxic, yet essential for life.

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