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Minireview

Metallochaperones, an Intracellular Shuttle Service for Metal Ions*

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Enzymes that employ transition metals as co-factors are housed in a wide variety of intracellular locations or are exported to the extracellular milieu. A key question then arises: how are specific metal co-factors transported to diverse locations and subsequently sorted into the correct metalloenzymes? The mechanisms by which these tasks are accomplished are just now being unraveled. A new family of soluble metal receptor proteins known as "metallochaperones" is emerging that act in the intracellular trafficking of metal ions. Although transition elements can be quite toxic, these metal receptors are not detoxification proteins; they clearly function in a "chaperone-like" manner, guiding and protecting the metal ion while facilitating appropriate partnerships. Here we will review the most recent advances in our understanding of copper metallochaperones and discuss mechanisms that may be relevant to other essential, yet potentially toxic, metal ions.

The Requirement for Copper Metallochaperones

The concept of copper metallochaperones is relatively new; prior to 1997, there were no established molecules that served this function. *In vitro*, most copper enzymes easily acquire their metal without an auxiliary protein. For example, the copper- and zinc-dependent enzyme superoxide dismutase (SOD1)[1] binds copper ions *in vitro* with an extraordinarily high affinity ($K_d \approx 10^{-15}$ M). Yet in a living cell, where *total* copper concentrations are in the micromolar range, SOD1 relies heavily upon an auxiliary factor for acquiring copper. This paradox is resolved by recent observations that establish an upper limit on the number of "free" copper ions available in the cytoplasm of an unstressed cell (1, 2).

Employing the yeast cell as a model, we have estimated the total cytoplasmic free copper concentration to be less than 10^{-18} M, which represents many orders of magnitude less than one atom of free copper per cell (1). In this usage, "free" copper is a thermodynamic term, which corresponds to aquo (hydrated) Cu(I) or Cu(II) complexes not coordinated by tight binding ligands such as amino acids or biopolymers. A similar conclusion regarding the scarcity of free intracellular copper ions can also be derived in kinetic terms; less than 0.01% of the total cellular copper becomes free in the cytoplasm during the lifetime of the cell (1). This apparent absence of free copper can be attributed to a wide variety of moderate and tight binding chelation sites in the cell including nonspecific small molecule interactions, as well as vesicular sites for concentration of the metal and specific copper proteins. This copper chelation capacity becomes even more potent when copper detoxification systems such as metallothioneins are induced. Despite this backdrop of cellular overcapacity for copper chelation, metallochaperones succeed in acquiring the metal and donating it to enzymes that need it (depicted in Fig. 1). Thus far, three distinct copper trafficking pathways have been described for copper, and these will be discussed independently.

Delivery of Copper to the Mitochondria

Cytochrome oxidase, a key mitochondrial enzyme in the respiratory chain, requires a total of three copper ions to be inserted into two subunits: a binuclear copper site protruding into the inner membrane space of the mitochondria and a mononuclear site buried within the inner membrane (3). It is not clear when or how these copper ions are inserted into the enzyme; however, among the host of assembly factors required for cytochrome oxidase activity, two proteins clearly have an effect on copper utilization. One of these, COX17, is a candidate metallochaperone.

COX17, originally discovered by Tzagoloff and co-workers (4, 5), is an 8.0-kDa protein bearing 6 cysteines that are conserved in the yeast and human proteins. Yeast COX17 localizes to both the cytosol and inner membrane space of the mitochondria, consistent with a role as a shuttle protein for delivering copper to mitochondria (6). Winge and co-workers (7) have shown that a copper-loaded COX17 can be isolated from *Escherichia coli* expression systems with Cu(I) ions bound in a sulfide and cysteine thiolate cluster. It is noteworthy that COX17 is predicted to require only four of the six conserved cysteinyl residues for metal coordination (7).

Copper incorporation into yeast cytochrome oxidase also requires the presence of SCO1, a mitochondrial inner membrane protein (8). SCO2, a homologue of SCO1, may also play a role in activation of cytochrome oxidase (9). SCO1 shares homology with subunit 2 of cytochrome oxidase, including two conserved copper-binding cysteinyl ligands (6, 9). It is possible that this region directly transfers copper to cytochrome oxidase, whereas COX17 may act as the shuttle protein to deliver copper to mitochondrial factors such as SCO1/SCO2. Until specific partnerships are established by biochemical or genetic means, alternative models cannot be excluded.

The ATX1 Pathway of Copper Delivery to the Golgi

We originally identified *ATX1* (anti-oxidant) in 1995 as a gene that afforded protection against oxidative damage in yeast (10). This anti-oxidant protection requires ATX1 overproduction but may not be physiologically relevant because activ-

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[1] The abbreviations used are: SOD1, superoxide dismutase; CCS, copper chaperone for SOD1; FALS, familial amyotrophic lateral sclerosis.

ity appears to result from stoichiometric, not catalytic, consumption of superoxide by Cu-ATX1 (11). Subsequently, ATX1 was found to specifically shuttle copper to an intracellular copper transporter located in the Golgi compartment of the secretory pathway (12–14). This copper transporter then pumps the metal into the lumen of the Golgi for insertion into copper enzymes destined for the cell surface or extracellular environment. Following identification of yeast ATX1, the human homologue was isolated by Gitlin and colleagues (15, 16) and has been denoted HAH1 or ATOX1.

The targets of copper delivery by ATX1 are P-type copper transporters that are also conserved among eukaryotes. These transporters are members of a large family of transporting ATPases that use energy from ATP hydrolysis to drive membrane transport of ions (reviewed in Ref. 17). Humans express two forms of this transporter, known as ATP7A and ATP7B. Inherited mutations in these copper transporters are responsible for Menkes syndrome and Wilson's disease, specific human disorders of copper metabolism (reviewed in Ref. 18). Yeast express a similar copper transporter known as CCC2 that is needed for activating the FET3 copper protein involved in iron uptake (19) (Fig. 1). A plant version of the copper transporter has been identified as RAN1 and functions together with an ATX1-like molecule to modulate plant growth in response to ethylene (20, 21).

The yeast ATX1 copper chaperone is known to bind a single metal ion via two cysteine residues present in the ATX1 amino acid sequence M*X*C*XX*C (where M is methionine, *X* is any amino acid, and C is cysteine) (13). The metal-binding site in ATX1 is flexible and accommodates additional bonding interactions with the Cu(I) atom, indicating that the protein readily allows changes in coordination number of the bound metal. This type of coordination environment was unprecedented in copper proteins but is now emerging as a common feature of copper trafficking proteins. Structural studies of ATX1, the copper chaperone for SOD1 (CCS), bacterial CopZ, and the metal-binding domains of the copper transporters that serve as targets for ATX1 have been reviewed elsewhere (22). In all cases, the polypeptides adopt a $\beta\alpha\beta\beta\alpha\beta$ fold with a similar tertiary structure in which two α-helices are superimposed on a 4-stranded β-sheet with a solvent-exposed metal-binding site. In ATX1, this fold has been shown to provide a tight Cu(I)-binding site and protects the metal center from both oxidants and from capture by excess competing thiols such as glutathione (13).

How does the Cu-ATX1 complex specifically recognize, dock with, and then transfer copper to the P-type metal transporter? ATX1 has been shown to physically interact with the ATX1-like domains of the copper transporter through a process involving copper ions and the M*X*C*XX*C copper site (11, 13, 23, 24). Docking of ATX1 also appears to involve electrostatic interactions between a positively charged face of ATX1 and negatively charged residues on the analogous segment of the copper transporter (11, 25) (Fig. 2). Once the metallochaperone has docked with its target, a cysteine from the acceptor domain is proposed to attack the Cu(I) center in ATX1. This initiates the formation and decay of a series of two- and three-coordinate Cu(I) centers in which the coordinated cysteines of ATX1 are sequentially replaced with cysteines of the copper transporter domains (13) (Fig. 2).

The thermodynamic gradient for metal exchange between ATX1 and CCC2 has been shown to be quite shallow, with a Cu(I) exchange equilibrium constant of 1.4 (26). Although this result reveals that ATX1 can release Cu(I) from a tight binding site, it begs the question of how the metallochaperone can provide sufficient flux of copper to the transporters. The present data suggest that ATX1 functions like an enzyme; it lowers the activation barrier for Cu(I) transfer with partner proteins and perhaps discriminates against non-partners as well. In support of this notion, copper is observed to rapidly equilibrate between ATX1 and CCC2, and the equilibrium is insensitive to an excess of copper scavengers that are common to the intracellular milieu. The ultimate driving force for copper transfer is then provided by other domains of the transporter, which employ ATP hydrolysis to remove Cu(I) to a separate thermodynamic compartment where multicopper oxidases and other apoproteins obtain the metal (26).

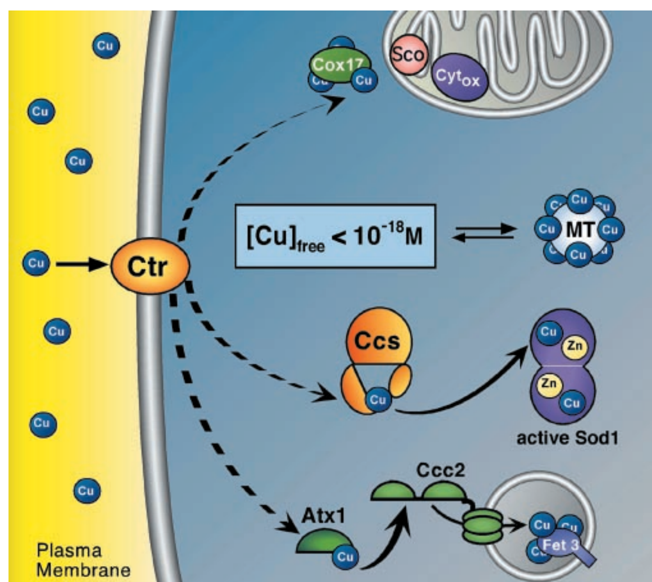

FIG. 1. **Copper trafficking pathways in eukaryotes.** Known pathways for the delivery of copper in yeast are depicted. Copper uptake, mediated in part by the cell surface copper transporter (*Ctr*) (47), is eventually deployed to mitochondrial cytochrome oxidase ((*Cyt_{ox}*) via a pathway involving Cox17 and Sco), to cytosolic SOD1 (via a pathway involving CCS), or to the copper transporter CCC2 and the multicopper oxidase Fet3 in the secretory pathway (involving ATX1). Cytosolic concentrations of free copper are typically maintained at exquisitely low levels ($<10^{-18}$ M) by metal scavenging systems including metallothioneins (*MT*) (1). *Dashed arrows* represent undefined pathways whereas *solid arrows* indicate established copper transfer steps discussed in the text.

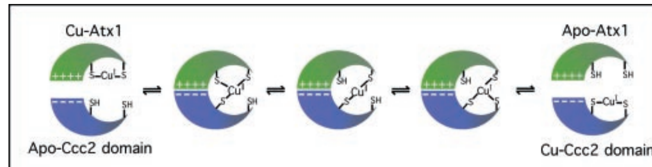

FIG. 2. **Proposed pathway for copper transfer from ATX1 to CCC2.** In this model, Cu(I) containing ATX1 docks with one of the apo metal-binding sites of CCC2 through electrostatic interactions involving positively charged residues on ATX1 and negatively charged amino acids on CCC2. Copper transfer (26) then occurs through an associative exchange mechanism as previously proposed (13).

Copper Delivery to Cytosolic Superoxide Dismutase

The target for CCS is a soluble copper- and zinc-requiring enzyme SOD1 (27). SOD1 protects cells against oxidative damage by scavenging toxic superoxide anion radicals through redox reactions at the bound copper ion. The *in vivo* insertion of copper into SOD1 requires a copper metallochaperone that was first identified in yeast as a gene involved in the lysine biosynthetic pathway, namely *LYS7* (28–30). Yeast mutants lacking LYS7 express a form of SOD1 that is essentially apo for copper (29, 31) but contains a single atom of zinc per dimer SOD1 (31).

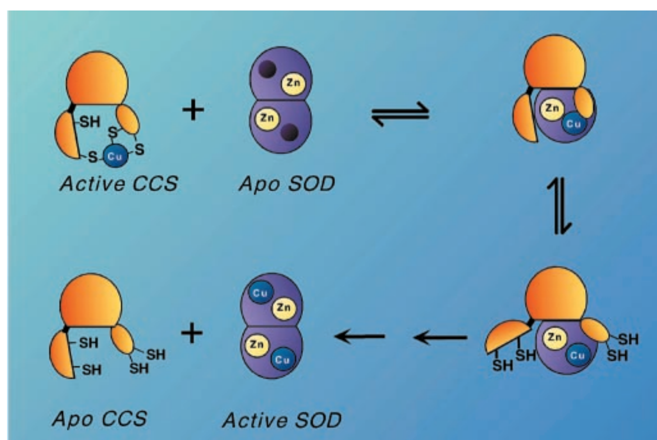

FIG. 3. **Capture and release model for copper transfer from CCS to SOD1.** The Cu(I) atom bridging Domains I and III of CCS is proposed to transfer to apo-SOD1 following formation of a heterodimeric or heterotetrameric complex (not shown) between CCS and SOD1.

The identification of the yeast metallochaperone for SOD1 quickly led to the cloning of the human homologue that had previously been identified as SOD4, a putative SOD isoform (29). Both yeast and human proteins have been denoted as CCS. Mice with targeted disruptions in CCS exhibit marked reductions in SOD1 activity, emphasizing the conserved requirement for CCS in activation of eukaryotic SOD1 (32).

CCS is the largest of copper metallochaperones identified to date. Whereas ATX1 and COX17 represent single domain proteins, CCS folds into three functionally distinct protein domains (Figs. 1 and 3) (33–36). The N-terminal Domain I of CCS bears striking homology to ATX1, including the M*X*C*XX*C copper-binding site. Surprisingly, however, a CCS molecule lacking this domain can still insert copper into SOD1 *in vivo*, provided the cell is not starved for copper (33). We therefore proposed that this ATX1-like domain is only needed to maximize CCS function under extreme copper-limiting conditions.

The central domain of CCS (Domain II) is homologous to its target of copper delivery, SOD1 (31, 34–38). With human CCS, this homology is so strong that a single mutation in Domain II is sufficient to turn CCS into a SOD-like molecule with superoxide scavenging activity (38). Domain II physically interacts with SOD1 (37) and is proposed to secure the enzyme during copper insertion. SOD1 normally exists as a homodimer, and formation of a transient heterodimer or a heterotetramer between SOD1 and CCS may precede copper transfer (34–36, 38) (Fig. 3).

The C-terminal Domain III of CCS is quite small (≈30 amino acids) yet is extremely crucial for activating SOD1 *in vivo* (33). This peptide is highly conserved among CCS molecules from diverse species and includes an invariant C*X*C motif that can bind copper (33). Domain III was disordered in the crystal structure; however, this domain is predicted to lie in the vicinity of the N-terminal Domain I (36, 39). Models have been proposed in which Domain III, perhaps in concert with the N-terminal copper site, directly inserts copper into the active site of SOD1 (33, 40). The mechanism of copper movement from an all-sulfur coordination environment (in CCS) to the all-nitrogen site in SOD1 remains an open issue.

It is noteworthy that human CCS may have some important relevance regarding the fatal motor neuron disease, familial amyotrophic lateral sclerosis (FALS). A subset of FALS cases results from dominantly inherited mutations in SOD1, and toxicity from the bound copper ion has been the designated culprit in certain models (41). Because FALS SOD1 mutants rely on the CCS (42) and mammalian CCS is abundantly expressed in neuronal tissue (43), CCS may play some part in the etiology of FALS.

Metallochaperones in Prokaryotes

Prokaryotes lack the intracellular compartmentalization that is typical of eukaryotes; thus organelle-specific carriers of metals such as COX17 may not be essential. Furthermore, bacteria express a copper and zinc SOD, but no prokaryotic homologue to CCS has been identified. However, a homologue to ATX1 (CopZ) has been described for enteric bacteria. CopZ was originally proposed to function as a copper transcription factor; yet based on its sequence and structural homology to ATX1, CopZ is a likely copper metallochaperone. Purified CopZ has the capacity to donate copper to the CopY transcription factor *in vitro*, and a model has been proposed in which this transfer of copper displaces the zinc ion needed for CopY binding to DNA (reviewed in Ref. 44). Other targets for CopZ, such as the copper efflux pump, have not been excluded.

Metallochaperones for Metals Other than Copper?

Although the bulk of knowledge on intracellular metal trafficking has emanated from studies of copper-based systems, it is likely that analogous cofactor trafficking pathways exist for other metals. Thus far, no eukaryotic chaperones for metals other than copper have been established; however, candidates for delivery of iron to the sites of iron-sulfur cluster assembly have been identified, such as the IscA family of proteins (45). Furthermore, prokaryotic nickel-binding proteins have been described that may facilitate the insertion of the metal into nickel-requiring enzymes, such as urease and cobalt dehydrogenase (reviewed in Ref. 46).

Conclusions

Redox-active transition metals such as copper present a dilemma to the cell; they are useful but dangerous cofactors. The metallochaperone proteins clearly do not function to protect the cell from metal toxicity. In fact, from studies in yeast, metallochaperones become critical for cell function only under copper limitation conditions (1, 4, 12). Instead, metallochaperones ensure the safe delivery of the metal ion to its proper intracellular destination and in the process protect the precious cargo from adventitious reactions and a multitude of alternative binding sites. How and where the chaperones themselves acquire copper remain a mystery, as such factors have yet to be identified.

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