Copper-binding motifs in catalysis, transport, detoxification and signaling

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Copper is required for many biological processes but is toxic at high cellular concentrations, so levels in the cell must be strictly controlled. Copper-binding motifs have been identified and characterized in many proteins. The way in which copper is coordinated by these motifs is important for the transport and distribution of intracellular copper and for the effective functioning of copper-dependent enzymes.

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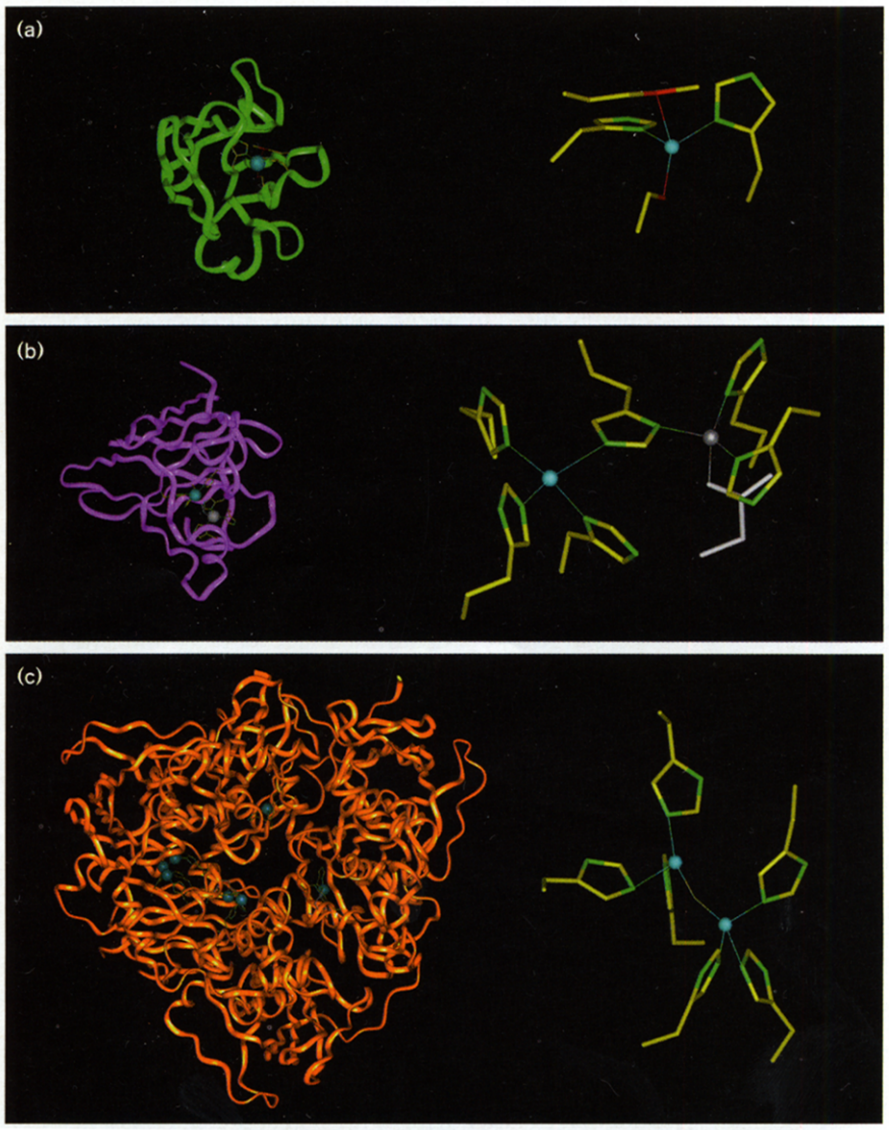
Introduction

The metal copper (Cu) was discovered in ~9000 BC. Since then, Cu has seen a number of uses, including in the creation of jewelry, as a material for cooking and storage vessels, as a medication for disease used by the ancient Greeks, as a key component of fungicides such as Bordeaux mixture (used to protect vineyards in France since the late 1800s) and currently as a potent conductor of electricity. Cu is very versatile and has a wealth of functions in biological systems, making it an essential requirement for all currently known life forms. As a cofactor for many biological reactions, Cu is important in processes as fundamental as respiration and as specialized as neurotransmitter maturation. However, the same chemistry that makes Cu essential also makes it a potent cytotoxin when homeostatic controls of the level of free Cu ions fail. Cells in all organisms must therefore strike a delicate balance so that sufficient Cu is acquired to carry out catalytic roles, yet the accumulation of Cu to abnormally high levels, which pose a threat to the cell, is prevented. In this review, we focus on how nature has developed sophisticated Cu-binding motifs to accomplish Cu-dependent enzyme catalysis, Cu transport, Cu detoxification and Cu-mediated signal transduction.

Copper coordination and catalysis

Many Cu-dependent enzymatic activities have been identified and biochemically characterized [1]. The precise coordination of Cu within protein structures plays a crucial role in the biochemical function of Cu-dependent proteins. Cu coordination geometry, stoichiometry and stability differ depending on the nature of the coordinating ligands and the oxidation state of the bound metal. Cu is a first row transition-metal ion which can assume two oxidation states, Cu(I) or Cu(II). In the Cu(I) oxidation state, the copper ion is a closed shell d^{10} transition metal ion and thus is silent in electron paramagnetic resonance (EPR) measurements [2]. As a soft Lewis acid, Cu(I) prefers a coordination number of two, three or four and soft Lewis base ligands such as thiolates or the sulfur of thioether moieties in tetrahedral four-coordinate or trigonal three-coordinate geometries. When Cu(I) is bound to polypeptides, these ligands are provided by the amino acids cysteine or methionine. In the Cu(II) oxidation state, the copper ion is a d^9 transition-metal ion that contains one unpaired electron in its outer shell, generally rendering Cu(II) active by EPR measurements with a preferred coordination number of four, five or six. Cu(II) is an intermediate Lewis acid so its range of polypeptide ligands is increased to include the imidizole nitrogen atoms of histidine, the carboxylate moieties of aspartate and glutamate, peptide backbone nitrogen and carbonyl groups, and the sulfur atoms of cysteine or

Figure 1



The three major classes of distinct Cu(II) metal centers found within proteins with diverse biological functions. **(a)** The crystal structure of the *Chlamydomonas reinhardtii* electron-transport protein plastocyanin (left) and an expanded view of the type 1 Cu center displaying distorted tetrahedral coordination geometry (right). In the expanded view, the histidine ligands (yellow/green) are shown on the left and right, and the methionine and cysteine ligands (yellow/red) are shown at the top and bottom, respectively, of the Cu atom (blue). **(b)** The crystal structure of the *Saccharomyces cerevisiae* Cu,Zn superoxide dismutase (left) and the expanded view of the dinuclear metal center with the type 2 Cu(II) atom (blue) in distorted square-planar coordination geometry and the Zn(II) atom (gray) in distorted tetrahedral coordination geometry. **(c)** The crystal structure of the *Homo sapiens* ferroxidase ceruloplasmin (left) and an expanded view of the two pyramidally coordinated type 3 Cu(II) atoms that are located within the trinuclear Cu center located on the left side of the ceruloplasmin structure. The coordinating ligands are histidine residues.

methionine. The coordination geometry of Cu(II) is typically square-planar with weakly bonded axial ligands. Interestingly, the large pool of potential coordinating ligands for Cu(II), and its proximity to neighboring Cu(II) ions or other paramagnetic metal centers, allows for three distinct classes of Cu(II) metal centers within proteins, which are denoted types 1, 2 and 3 [2].

Plastocyanin, a key photosynthetic electron-transport protein [3], is a representative Cu protein with a type 1 Cu center, characterized by two histidine imidazole nitrogen ligands and two sulfur ligands from cysteine and methionine coordinating Cu(II) in a distorted tetrahedral geometry (Figure 1a). In plastocyanin, the type 1 Cu center facilitates electron transfer between the cytochrome b_6f complex and the photosystem I complex in higher plants, algae and cyanobacteria [4]; its position in the photosynthetic electron-transport chain is analogous to the position of cytochrome c in the electron-transport chain in mitochondria. Type 1 Cu centers have EPR properties that are characteristic of a highly covalent Cu(II) and a visible absorbance maximum (λ_{max} ~650 nm), giving purified type 1 Cu proteins a distinctive blue appearance (blue copper protein).

A harmful side product of respiration is generated by the one electron reduction of dioxygen to produce a superoxide anion ($O_2^{-\bullet}$) [5]. The enzyme copper, zinc superoxide dismutase (Cu,Zn SOD), which disproportionates the superoxide anion and therefore plays a key role in protection against oxidative stress, typifies a protein with a type 2 Cu center. The Cu ion in Cu,Zn SOD is essential for the enzyme's extremely efficient catalytic disproportionation of the superoxide anion to hydrogen peroxide and dioxygen.

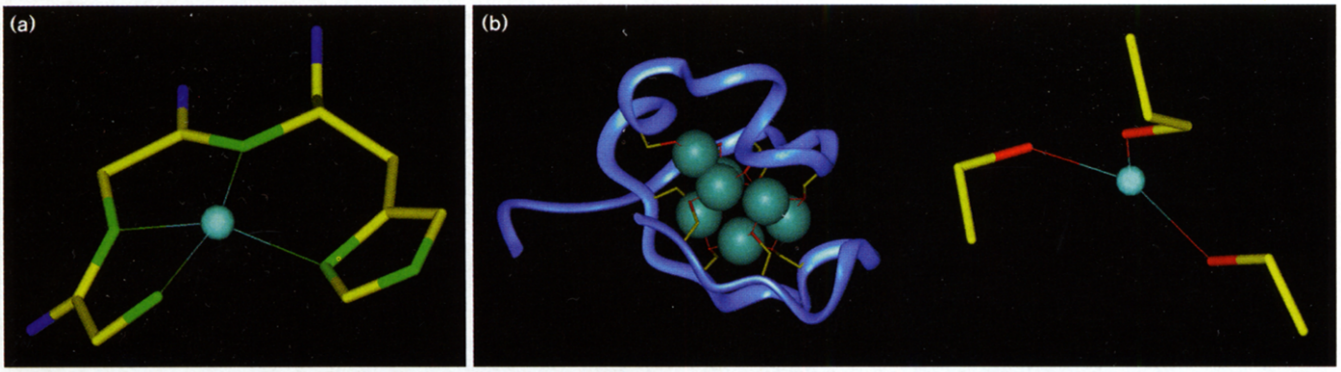
In the Cu,Zn SOD of baker's yeast, *Saccharomyces cerevisiae*, the Cu(II) is coordinated in a distorted square-planar geometry by four histidine imidazole nitrogen atoms, with a fifth axial coordination position facing the solvent (Figure 1b) [6]. The Zn(II) ion is coordinated by three histidine sidechains and one aspartate sidechain in a distorted tetrahedral geometry, with one of the histidine residues bridging the single Cu and Zn atoms. The potential role of the superoxide radical in causing cancer, aging and neurodegenerative disease has recently been underscored by the observation that mutations in the human Cu,Zn SOD gene leading to amino acid substitutions were identified as one cause of familial amyotropic lateral schlerosis, a fatal neurodegenerative disease resulting in motor neuron loss [7]. Subsequent studies have shown that these mutations in Cu,Zn SOD do not markedly reduce SOD activity [8]. Rather, consistent with the dominant nature of the mutant alleles, the mutations appear to confer on the enzyme a function other than that of superoxide disproportionation. Whether these mutations operate simply by enhancing the rate of the inherent peroxidase activity, whereby the enzyme uses the hydrogen peroxide generated from the dismutase reaction in a side reaction that generates hydroxyl radicals [9], via the nitration of proteins [10] or other mechanisms, remains to be determined.

The single most abundant form of Cu in human serum is bound to a 132 kDa glycoprotein, ceruloplasmin [1]. Ceruloplasmin, synthesized in hepatocytes, is a Cu-dependent Fe(II) oxidase that contains seven Cu atoms incorporated during its biosynthesis and maturation on the intracellular pathway that leads to its secretion (Figure 1c) [11]. Although Cu does not influence the biosynthesis or secretion of ceruloplasmin, failure to incorporate Cu into ceruloplasmin results in an unstable protein and a loss of the ferroxidase activity which is essential for Fe mobilization. Patients lacking ceruloplasmin (suffering from aceruloplasminemia) accumulate Fe in the liver and in a number of other tissues in the body, resulting in damage to these tissues, presumably due to Fe-mediated hydroxyl radical generation [12]. Ceruloplasmin is an unusual Cu protein because it contains type 1, type 2 and type 3 Cu centers. Type 3 Cu centers are classified by their silent nature in EPR analysis, caused by antiferromagnetic exchange coupling between two neighboring paramagnetic metal centers (Figure 1c) [2]. The type 3 Cu center in ceruloplasmin is characterized by the exclusive use of histidine residues as the Cu(II) ligands. Thus, although the activity of Cu-dependent enzymes can serve as an indicator of biologically available Cu in cells, Cu within Cu-dependent enzymes can serve as a useful probe of the coordination environment and oxidation state within these proteins. In addition to the Cu enzymes described here, a number of other important Cu-dependent enzymatic activities have been studied. Such enzymes include dopamine β-hydroxylase and peptidyl glycine α-amidating enzyme (both of which are involved in neurotransmitter biosynthesis), lysyl oxidase (an enzyme that cross-links collagen and elastin in connective tissue maturation), laccase (a plant enzyme catalyzing phenol oxidation), tyrosinase (an important enzyme in melanin biosynthesis), and blood clotting factors V and VIII [1].

The coordination of Cu ions by proteins, in either an exchangeable form or as a tightly sequestered metal ion, is largely determined by the coordination ligands and geometry. Furthermore, the redox potential of Cu within a polypeptide can be altered substantially depending on the available coordinating ligands, how strongly the polypeptide chain controls the coordination geometry of the metal center, and the dielectric environment of the metal center. For example, an environment that has a tetrahedral coordination of Cu(II) would promote the transition to the Cu(I) oxidation state and consequently raise the redox potential of the metal. Additionally, a Cu(II) coordinated with thiolate or thioether ligands outside its preferred coordination geometry would have its redox potential further altered. Proteins have evolved to utilize these and other principles of coordination to effectively fine tune the redox potential and stability of the bound Cu to produce the desired function of the protein. Examples of Cu-binding motifs discussed below underscore the influence of the coordination environment on Cu stability and reactivity.

Extracellular protein ligands involved in Cu transport across biological membranes, or intracellular proteins that function to distribute Cu, probably coordinate Cu ions in a less labile, more mobilizable fashion than Cu enzymes. On the other hand, proteins that sequester Cu to prevent redox chemistry that leads to toxicity predominantly function through tenacious Cu ligands. The amino-terminal Cu(II)- and Ni(II)-binding (ATCUN) motif, found in serum albumin and other polypeptides, is an example of a Cu(II)-binding motif that shows high affinity and specificity for Cu(II) or Ni(II), but is also capable of facile metal exchange with appropriate receptor ligands [13]. The ATCUN motif in proteins binds Cu(II) in a slightly distorted square-planar geometry using three nitrogen atoms from the peptide backbone (one of these is derived from the free amino terminus) and an imidazole-ring nitrogen (Figure 2a). Although the Cu-coordination geometry and the use of nitrogen ligands by proteins utilizing the ATCUN motif is reminiscent of the coordination of type 2 Cu centers, ATCUN-motif proteins actually exhibit a visible absorbance maximum (λ_{max} 525 nm), suggesting that the Cu bound to the ATCUN motif belongs to a distinct class of metal centers. Although only about 5% of the total serum Cu is complexed with albumin, Cu(II)–albumin is thought to be the major transport form of Cu(II) in the blood [1]. It has also been proposed that the ATCUN motif plays a role in preventing Cu toxicity. It is interesting that dog serum albumin lacks

Figure 2



Two key components of the Cu homeostatic pathways involved in Cu detoxification and Cu distribution. **(a)** The structure of the Gly–Gly–His tripeptide coordinating an exchangeable Cu(II) atom as an example of the ATCUN motif found in the serum albumin of mammals. **(b)** The averaged nuclear magnetic resonance (NMR) structure of the *S. cerevisiae* metallothionein Cup1, containing seven Cu(I) atoms coordinated tightly into the folds of the polypeptide backbone (left) and an expanded view of one of the trigonally coordinated Cu(I) atoms. The coordinating ligands are cysteine residues.

the ATCUN motif, perhaps contributing to the acute sensitivity of many dogs to Cu toxicity. The ATCUN motif therefore has the Cu(II) coordination strength to maintain the complexed Cu, yet allow appropriate metal-ion exchange for Cu transport and distribution.

Copper toxicity

The destructive potential of Cu towards cellular macromolecules can be attributed to two properties of the metal. The first is the ability of Cu to change its redox state within the cell [Cu(I)↔Cu(II)]. This redox activity permits Cu to catalyze Fenton-like reactions, in which Cu(I) reacts with hydrogen peroxide (H_2O_2) to form Cu(II) and the highly reactive and therefore damaging hydroxyl radical (·OH) [5]. The second cytotoxic property of Cu is its ability to interact nonspecifically with amino acid sidechains such as those derived from cysteine, methionine and histidine, resulting in the displacement of existing metal ions and/or the misfolding of proteins. This has been well documented for the estrogen receptor, a hormone-responsive DNA-binding transcription factor that normally coordinates two Zn atoms for proper structural configuration of the DNA-binding domain [14,15]. The ability of Cu to displace Zn in the estrogen receptor is due in part to the similar ligand preferences of the two metals and is also likely to be a result of the ability of Cu to form a more stable complex with these ligands. The consequence of the essential yet toxic nature of Cu is that all cells must have homeostatic mechanisms that maintain Cu at a level required for Cu-dependent enzymes but that also prevent the accumulation of Cu to toxic levels.

One of the most efficient ways of preventing Cu toxicity, under conditions of extreme Cu excess, is to sequester Cu in a nonexchangeable form. When Cu is in excess of physiological requirements, prokaryotic and eukaryotic cells induce the biosynthesis of small cysteine-rich proteins known as metallothioneins, which bind Cu atoms cooperatively and tenaciously [16]. The cytoplasm of most eukaryotic cells is a reducing environment and therefore the predominant form of the free Cu in the cell would be Cu(I). In this oxidation state, excellent coordination ligands to use for chelation of the Cu are cysteine thiolates, which are arranged in the metallothioneins in Cys–X–Cys and Cys–X–X–Cys configurations (where X is any amino acid). Mammalian metallothioneins contain two domains, an α domain and a β domain, and although structural data is available for metallothioneins containing Zn and Cd [17], the structure of a mammalian Cu metallothionein has remained elusive. The limited data available for the Cu clusters in a mammalian Cu metallothionein suggest that the protein contains 12 Cu(I) atoms, with six Cu(I) atoms bound per domain with an apparent trigonal coordination geometry.

The *S. cerevisiae* Cup1 metallothionein contains a single domain that has a structure resembling the β-domain of mammalian metallothioneins, and has served as a useful model for understanding Cu(I) coordination in Cu detoxification and signaling proteins. Spectroscopic studies have identified the coordination of Cu(I) in a polynuclear cluster that has luminescent properties under anaerobic conditions, indicating a solvent-shielded environment [18]. The nuclear magnetic resonance (NMR) structures of Cu metallothionein and Ag metallothionein from *S. cerevisiae* were solved recently [19], providing the first three-dimensional structural information on the mechanism of Cu(I) coordination in metallothioneins (Figure 2b). These studies have elegantly demonstrated that although the apo-Cup1 has no regular secondary structural features, the Cu(I)- or Ag(I)-metallated Cup1 protein forms two parallel loops that enfold a central Cu cluster. Within this cleft

reside seven Cu atoms, largely in trigonal coordination geometry. Although there are rather subtle differences between the three-dimensional structures of Cu(I)- and Ag(I)-reconstituted Cup1, it is clear that Ag(I)-substituted proteins are excellent experimental tools for probing the structure of Cu(I)-binding proteins.

The transport of copper into cells and its intracellular distribution

In mammals, Cu is absorbed in the stomach and duodenum where it is thought to be transported to the liver by serum proteins that include albumin, a Cu protein known as transcuprein, and perhaps other soluble factors such as amino acids [1]. Normal physiological Cu levels in humans are maintained through a balance between the absorption and distribution of Cu, and its biliary and urinary excretion. The necessity for precise Cu homeostatic control mechanisms in biological systems is underscored by the occurrence of severe human diseases of Cu homeostasis.

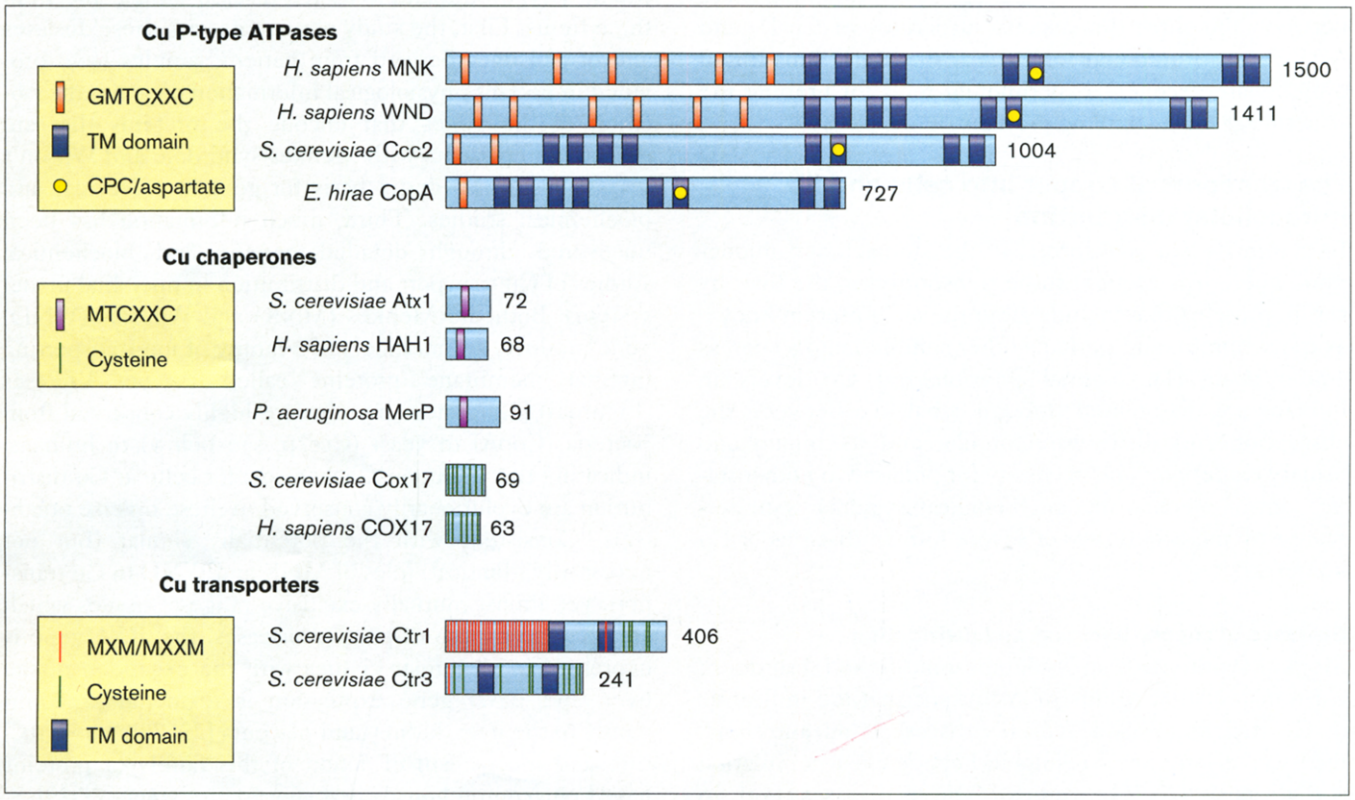
Diseases of copper transport and distribution

Menkes syndrome is an X-chromosome-linked disorder of Cu transport that results in symptoms that are indicative of Cu deficiency, and Wilson's disease is an autosomal recessive disorder that results in hepatic Cu accumulation and toxicosis [20,21]. Studies of these diseases, both in the laboratory and in the clinic, have been a rich source of information about Cu transport and distribution in mammals, and have stimulated mechanistic and structural studies of proteins that play key roles in Cu transport and distribution. In patients with Menkes syndrome, Cu uptake and excretion from the liver are quite normal but there is a severe defect in the intestinal absorption of Cu. Although greater than normal Cu concentrations are found in most non-hepatic tissues in Menkes patients, Cu levels in the brain, liver and serum are markedly reduced. The inadequate level of biologically available Cu in these tissues results in a severe reduction in the activities of the Cu-requiring enzymes cytochrome oxidase, lysyl oxidase, tyrosinase and dopamine β-hydroxylase. As a consequence of these and other aspects of Cu-homeostatic defects, Menkes patients have a number of clinical and biochemical phenotypes, including neurological degeneration, poor temperature regulation, kinky hair, propensity for arterial rupture and, typically, mortality in early childhood. In contrast to those with Menkes syndrome, patients with Wilson's disease have normal intestinal and hepatic Cu uptake, but biliary excretion of Cu and the incorporation of Cu into ceruloplasmin (the multi-Cu ferroxidase involved in Fe mobilization) are severely impaired. As a result, high levels of Cu accumulate predominantly in the liver and brain, resulting in hepatic cirrhosis and neurological degeneration. Menkes syndrome and Wilson's disease therefore both represent diseases of Cu transport or distribution, but they largely affect distinct tissues.

Major advances in our understanding of the etiology of Menkes syndrome and Wilson's disease have come from three fronts. First, the study of patients with these diseases and of cell lines derived from patient samples have provided important physiological information. Second, the isolation of the genes that encode the proteins that are defective in patients with Menkes syndrome and Wilson's disease have provided a basis for genetic, molecular and biochemical studies. Third, much information has been uncovered through detailed genetic and biochemical studies of Cu transport and distribution in microbial model systems. Both the Menkes (MNK) and Wilson's (WND) gene products are members of a family of ion-transporting integral membrane proteins called P-type ATPases (Figure 3) [22]. These proteins are highly conserved from bacteria (CopA) to yeast (Ccc2p, see below) to humans, indicating that common mechanisms for cellular Cu distribution are evolutionarily conserved in these diverse organisms. Consistent with the potentially similar (but not necessarily identical) roles of MNK and WND in Cu transport, yet almost mutually exclusive range of tissues which are affected by the respective diseases, the *MNK* gene is expressed in all human tissues with the exception of the liver, and *WND* gene expression is restricted predominantly to the liver, kidney and placenta [23–28]. The structure and mechanism of action of this family of proteins have been comprehensively reviewed elsewhere [22,29].

Figure 3 highlights the basic features of a representative group of Cu-transporting P-type ATPases. These include one or more potential heavy metal-associated (HMA) motifs at the amino terminus (Gly–Met–Thr–Cys–X–X–Cys), several hydrophobic membrane-spanning domains, one of which contains a conserved Cys–Pro–Cys (CPC) motif, an ATP-binding domain, aspartyl kinase and phosphatase domains and an invariant Asp–Lys–Thr–Gly–Thr (DKTGT) motif, in which the aspartyl residue (D) is required for the formation of an acylphosphate intermediate. Although structural and biochemical studies of Cu-transporting P-type ATPases are at an early stage, it is thought, on the basis of studies of related molecules, that the acylphosphate intermediate is formed by the transfer of the γ-phosphate of ATP to aspartate in the conserved DKTGT motif. The dephosphorylation of this residue by the phosphatase domain provides the energy to trigger a conformational change in the protein, thereby resulting in the transport of Cu atoms that are bound to the highly conserved metal-binding motifs located at the amino terminus of the proteins through a channel, possibly formed by the membrane-spanning domains. Do these amino-terminal domains bind Cu ions, what oxidation state of Cu is bound, what are the ligands and what is the stoichiometry of this binding? Recent studies have demonstrated that the amino-terminal putative metal-binding domains bind Cu preferentially and cooperatively, with a stoichiometry of one Cu atom per heavy metal-binding module [30] (B. Sarkar,

Figure 3



Primary structural comparison of key proteins involved in Cu transport and distribution in bacteria, yeast and humans. The polypeptide lengths are drawn to scale and putative Cu coordination motifs that are conserved among species are indicated in the keys to the left of each set of proteins using single-letter amino acid codes. Although the *Pseudomonas aeruginosa* MerP protein is not involved in Cu homeostasis, the MTCXXC motif of Atx1 was discovered as a result of its homology to MerP. *E. hirae, Enterococcus hirae;* TM domain, transmembrane domain.

personal communication). Although the thiolates of cysteine and methionine would be expected to coordinate Cu, perhaps as Cu(I), structural and biochemical work currently under way in a number of laboratories, in combination with established information on Cu coordination in other proteins and model coordination compounds, should provide more detailed structural and mechanistic answers.

Copper transport

One level at which Cu homeostasis is tightly controlled is through the regulation of its transport into cells. In mammals, relatively little is currently known about the precise components involved in Cu transport and mechanisms by which Cu is transported across the plasma membrane into cells [31]. Studies in bacteria and yeast have demonstrated that Cu uptake and distribution require proteins that have conserved putative metal-binding domains. A number of membrane proteins that participate in Cu transport, of both chromosomal and plasmid origin, have been identified in prokaryotic organisms. One of the best-characterized systems for Cu transport is derived from the Gram-positive bacterium, *Enterococcus hirae*. In *E. hirae*, two membrane P-type ATPases, CopA and CopB, are involved in Cu transport into the cell under limiting Cu conditions and Cu export under conditions of Cu excess, respectively [22]. Indeed, the *E. hirae* CopB ATPase is the only ATPase to date that has been biochemically demonstrated to transport Cu in reconstituted membrane vesicles *in vitro* [32]. The less well-characterized CopA has a single amino-terminal presumptive Cu-binding motif that is conserved in the MNK, WND and Ccc2 proteins (Figure 3). Although CopA activity has yet to be reconstituted in membrane vesicles *in vitro*, the requirement of *E. hirae* cells for CopA for the transport of Ag(I) would suggest, on the basis of the similar electronic properties of Cu(I) and Ag(I), that other members of the Cu P-type ATPase family may bind Cu(I) via the amino-terminal conserved motif. Studies in simpler model systems therefore are expected to continue to provide a comprehensive mechanistic understanding of the components involved in Cu transport in higher organisms.

Cu transport into eukaryotic cells is best understood in *S. cerevisiae*, in which early experiments on Cu-uptake

suggested the presence of ATP-dependent high affinity Cu-transport mechanisms [33]. Prior to uptake, Cu(II) is thought to be reduced from Cu(II) to Cu(I) by the extracellular Fe(III)/Cu(II) reductases Fre1 and Fre2 encoded by the *FRE1* and *FRE2* genes [34,35]. Although the rationale for Cu reduction is not clear, one possibility is that Cu(I) has greater specificity for extracellular ligands from Cu-transport proteins in the plasma membrane than Cu(II). High affinity Cu transport in *S. cerevisiae* is mediated by two integral membrane proteins encoded by the *CTR1* and *CTR3* genes [36,37]. The discovery of *CTR1*, through the characterization of yeast mutants defective in Fe transport, led to a wonderful mechanistic understanding of a long-known but poorly understood link between Cu availability and Fe mobilization. Most yeast strains in which the *CTR1* gene has been inactivated (*ctr1Δ* cells) have several phenotypes that are all attributable to a defect in high affinity Cu uptake [36,38]. These include poor growth on low Cu media, respiratory deficiency (due to the requirement for Cu as a cofactor of cytochrome oxidase in respiration), sensitivity to oxidative stress (due to low Cu,Zn SOD activity), and the inability to induce transcription of the *CUP1*-encoded metallothionein due to defective delivery of Cu to the Ace1p metalloregulatory transcription factor (see below). Furthermore, *ctr1Δ* cells are defective in high affinity Fe transport because they are unable to supply Cu to Fet3, a multi-Cu/Fe(II) oxidase required for Fe transport, in the secretory pathway [39]. The Ctr1 protein forms oligomers *in vivo* and contains two potential membrane-spanning domains and a large number of repeats of a putative Cu-binding motif Met–X–Met or Met–X_2–Met within its amino terminus, a hydrophilic region of the protein thought to reside in the periplasmic space between the yeast plasma membrane and the cell wall. Although it is currently not known whether this motif is directly involved in Cu(I) coordination, the methionyl sulfur atoms in the Ctr1 amino terminus would provide a high concentration of excellent ligands for Cu(I) coordination and transport. The recent characterization of a human cDNA encoding a Ctr1p functional homolog will provide an important tool for understanding Cu transport in mammalian cells [40].

A second high affinity Cu transport protein, Ctr3, was identified by the isolation of a yeast mutant that could suppress the Cu deficiency defects associated with a *ctr1Δ* strain [37]. Interestingly, expression of the *CTR3* gene in most laboratory *S. cerevisiae* strains is repressed by the insertion of a Ty2 transposable element between the *CTR3* basal promoter and the start site of transcription. In the suppressor strain the transposon was largely excised, restoring expression of the *CTR3* gene. Ctr3p is a small cysteine-rich integral membrane protein (241 amino acids) that contains 11 cysteine residues, of which three pairs are arranged in a potential metal-binding Cys–X–X–Cys or Cys–Cys motif (Figure 3). Although the Ctr1 and Ctr3 proteins can function independently in high-affinity Cu transport, the expression of both proteins allows maximal Cu uptake and growth under Cu-limiting conditions [37].

Intracellular distribution

Recent studies in *S. cerevisiae* have identified several proteins involved in intracellular Cu distribution and demonstrate their remarkable conservation of structure and function in humans. Studies have shown that a functional *CCC2* gene, encoding a homolog to the MNK and WND proteins of humans, is required for incorporating Cu into the Fet3p multi-Cu/Fe(II) oxidase, which forms a complex with the Ftr1p Fe permease to carry out high affinity Fe uptake at the plasma membrane [41,42]. As Fet3p and Ftr1p are secreted proteins, it is likely that, like the MNK and WND proteins, the yeast Ccc2p may be localized to the *trans*-Golgi network. The Ccc2 protein has all the features of putative Cu-transporting P-type ATPases shown in Figure 3, including two copies of the HMA motif in the amino-terminal domain.

How does Cu, once transported into the cell, efficiently reach its appropriate destination in Cu proteins without participating in harmful side reactions that generate hydroxyl radicals? Within the cell, Cu appears to be distributed to Cu-requiring proteins through the use of so-called Cu chaperones. An experiment in which a gene present on a multicopy plasmid suppressed oxygen toxicity in yeast cells lacking superoxide dismutase, uncovered the first eukaryotic Cu chaperone, Atx1p [43]. Atx1p is a 73 amino acid cytosolic protein that shows homology to the conserved metal-binding motifs found in the amino terminus of CopA in *E. hirae* and the well-characterized mercury-binding periplasmic protein (MerP) from *Pseudomonas aeruginosa* [44]. Like MerP, Atx1p has a single copy of the Met–X–Cys–X–X–Cys sequence and has homology along its entire sequence to several as yet uncharacterized open reading frames found in plants and worms. The binding of a single Hg^{2+} to the Met–X–Cys–X–X–Cys domain of MerP would predict that a single Cu(I) atom may be coordinated at this site in the Atx1 protein. The nature of the Cu site in Atx1p was recently revealed to be an unusually stable mononuclear Cu(I) site coordinated by two cysteine thiolate ligands and possibly one methoinine thioether ligand (J.E. Penner-Hahn, T.V. O'Halloran and V.C. Culotta, personal communication).

A recent model, based upon genetic epistasis experiments, suggests that Atx1p gives Cu to Ccc2p in yeast and that, in the absence of Atx1p, the yeast has an Fe-starvation phenotype caused by an inability to deliver Cu ions to the Fet3p multi-Cu/Fe(II) oxidase in the secretory apparatus [45]. The unusual stability of the Cu(I) site in Atx1p is thought to allow Cu(I)–Atx1p to reach its target, Ccc2p, without

undergoing oxidation to Cu(II), and a ligand exchange mechanism has been proposed for the transfer of Cu from Atx1p to Ccc2p. Overproduction of Atx1p from a high copy plasmid could therefore rescue the oxidative stress phenotype of a *sod1Δ* strain (a strain in which the gene encoding Cu,Zn SOD is inactivated), through Atx1p binding Cu and facilitating its sequestration within the secretory network. Importantly, a human Atx1p homolog (HAH1) has recently been identified; it shares structural similarity to the Atx1 and MerP proteins, and it complements the defects associated with a yeast *atx1Δ* gene deletion [46]. Like Atx1p, the human HAH1 protein may function to bind Cu(I) and supply Cu to the MNK and WND proteins in humans.

The assembly of the multiple subunits of cytochrome oxidase, a pivotal component of the mitochondrial respiratory pathway, is a complex process that involves a number of accessory factors. Because cytochrome oxidase is both an Fe and a Cu protein, the incorporation of these metals into the holoenzyme is essential for correct assembly. A Cu chaperone from yeast cells, Cox17p, has recently been identified by genetic experiments and has been shown to play an essential role in the delivery of Cu to the mitochondria for respiratory function [47]. Inactivation of the *COX17* gene results in loss of cytochrome oxidase activity due to an assembly defect and a corresponding respiratory deficiency. Cox17p is a 69 amino acid soluble cytosolic protein that contains seven cysteine residues (Figure 3). A Cys→Tyr mutation at codon 57 renders the Cox17 protein nonfunctional as determined by the lack of incorporation of Cu into cytochrome aa_3, a failure to assemble the cytochrome oxidase complex in mitochondria and a corresponding respiratory deficiency. In support of the role of Cox17p in the mobilization of Cu into cytochrome oxidase, the respiratory-deficient phenotype of *cox17* deletion mutants can be corrected by supplementing the growth media with high concentrations of Cu. Furthermore, overexpression of the Ctr1 protein in these mutants lowers the concentration of Cu needed for remediation of the respiratory defect nearly fourfold. As with Atx1p, a human complementary DNA encoding a structural and functional Cox17p homolog (hCOX17) has recently been isolated (Figure 3) [48]. It is interesting that yeast *cox17* mutants are not defective in Cu,Zn SOD activity, nor are *atx1* mutants completely defective in respiration. These observations further underscore the notion that Cu is delivered to different targets by distinct carriers. Whether each Cu-requiring protein has its own system for delivery, or whether different Cu chaperones have specific sets of targets due to distinct specificity determinants, are exciting questions for investigation. The high degree of structural and functional similarity between the yeast and human components involved in intracellular Cu distribution, and perhaps other molecules involved in Cu transport into the cell, indicates that defined Cu-binding motifs are conserved through evolution.

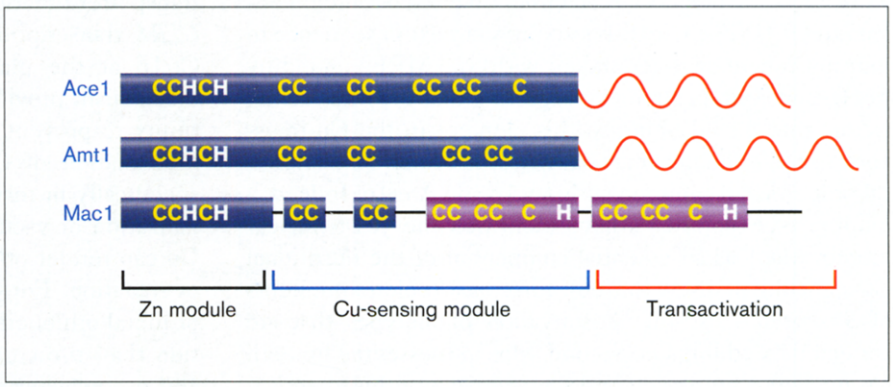
Regulation of transcription and post-translational events by copper

Although it is clear that organisms ranging from bacteria to mammals must have the capacity to sense and respond to changing environmental Cu levels, relatively little is known about the sensors used to accomplish this task in higher eukaryotic organisms. Bacterial and yeast cells have provided valuable insight into Cu sensing and signaling through the study of Cu metalloregulatory transcription factors (Cu-MRTFs). The two most extensively studied members of the yeast Cu-MRTF family are Ace1p and Amt1p from *S. cerevisiae* and the opportunistic pathogenic yeast *Candida glabrata*, respectively [49–51]. Ace1p activates transcription of the *CUP1* and *CRS5* metallothionein genes, and the Cu,Zn SOD gene (*SOD1*) in response to Cu concentrations that, although nontoxic, are clearly well above the range needed for robust cell growth [52]. *C. glabrata* has a metallothionein gene family consisting of *MT1*, *MT1Ia*, and *MT1Ib* and as yet unidentified members that are transcriptionally activated by Amt1p in response to micromolar Cu concentrations [53]. Interestingly, the *ACE1* gene is constitutively expressed and Ace1p appears to be constitutively localized to the nucleus. In contrast, *AMT1* mRNA levels increase nearly 20-fold above basal level in response to increases in environmental Cu concentrations via transcriptional autoregulation of the *AMT1* gene by Amt1p. Furthermore, rapid and robust autoregulation of the *AMT1* gene is essential for Cu resistance, because basal levels of Amt1p are insufficient to fully activate the metallothionein family of target genes and confer normal levels of Cu tolerance [54]. Moreover, the *AMT1* gene promoter is packaged into a nucleosomal structure that is slightly distorted adjacent to the metal regulatory element (MRE), allowing rapid access of Amt1p to its binding site in chromatin in response to elevated Cu levels [55]. Although Ace1p and Amt1p are essential components for sensing and rapidly responding to Cu to activate expression of the detoxification genes, *C. glabrata* has an added regulatory level, perhaps to limit unnecessary metallothionein synthesis, probably because opportunistic pathogens often inhabit sparse nutritional environments.

How do yeast Cu-MRTFs sense Cu and how is this information translated into a biochemical signal? Extended X-ray absorption fine structure (EXAFS) analysis has demonstrated that Cu coordination in Cu-MRTFs is similar to that in the Cu-coordinated yeast metallothionein, Cup1p (Figure 2b) [56,57]. Both Ace1p and Amt1p coordinate four Cu(I) atoms per polypeptide in predominantly trigonal geometry, via cysteine sulfur atoms. Cu(I) coordination occurs within the amino-terminal cysteine-rich DNA-binding domain (~110 amino acids), with residues 43–110 shown to be sufficient for the tetra-Cu cluster formation and high affinity sequence-specific DNA binding (Figure 4, Cu-sensing module) [58,59]. The identification of Cu(I)–S charge transfer absorbance in Cu-MRTFs, and

Figure 4

A primary structural and functional domain comparison of the yeast metalloregulatory transcription factors Ace1, Amt1, and Mac1. The cysteine and histidine residues and the Zn(II)-binding module of each protein are indicated. Note that all of the cysteine residues in the Cu-sensing module are indicated for Ace1 and Amt1. The terminal cysteine in Ace1 is not thought to be involved in the coordination of the tetra-Cu(I) cluster, however. The repeated $CXCX_4CXCX_2CX_2H$ motif in the carboxyl terminus of Mac1 (indicated in violet) is thought to be involved in both Cu sensing and transcriptional activation.



NMR, atomic absorption and electrospray mass spectrometry studies have confirmed the presence of a tetra-Cu(I) cluster within the DNA-binding domains of both Ace1p and Amt1p. Furthermore, both Ace1p and Amt1p show luminescence, much like Cu-metallothioneins, that has been attributed to the presence of Cu(I)–thiolate clusters that are shielded from solvent. Although no three-dimensional structure has been established for either Ace1p or Amt1p, partial proteolysis and circular dichroism experiments clearly demonstrate that the coordination of Cu(I) within the DNA-binding domain of these transcription factors drives large conformational changes, rendering them competent for high affinity sequence-specific DNA binding as monomeric proteins [60]. Cu is bound by Ace1p and Amt1p in a cooperative fashion, which is a desirable trait for a toxic Cu(I) sensor and suggests that Cu-clusters are formed, similar to those in Cup1p.

Given that Ace1p and Amt1p share 50% amino acid sequence identity in their metal-coordination/DNA-binding domains, it is not surprising that both transcription factors bind equivalent amounts of Cu(I) and share very similar target DNA elements. Upon activation by Cu(I), both Ace1p and Amt1p bind to DNA sequences 5′-TXXXGCTG-3′, known as MREs [53,60]. The core sequence 5′-GCTG-3′ is contacted in the major groove, and the AT-rich 5′ sequences are contacted in the minor groove [61]. Additional major-groove interactions have been noted for Ace1p and Amt1p just upstream of the 5′-end of the AT-rich minor-groove interactions [62]. The DNA sequences containing these upstream contacts are not strictly conserved among the MREs in yeast, however, and may represent additional contacts made in a subset of binding sites for Cu-MRTFs. Both Ace1p and Amt1p bind to DNA as monomers yet contact both the major and minor grooves of target DNA sites simultaneously, suggesting that the DNA-binding domains of these proteins may have at least two subdomains. Indeed, the putative minor-groove-binding domain of yeast Cu-MRTFs is found within the amino-terminal 40 amino acid residues and contains a Gly–Arg–Pro tripeptide, which bears striking homology to the minor-groove-binding domain of the human high mobility group protein HMG-I(Y) (Figure 4, Zn module) [61]. Interestingly, recent studies have identified the presence of a single Zn(II) atom, coordinated by cysteine and histidine ligands, within the first 40 amino acids in the DNA-binding domains of both Ace1p and Amt1p [58,59]. A Zn(II)-coordination site in Amt1p closer to the amino-terminus than to the minor-groove-binding domain, would therefore place the minor-groove-binding domain between a region structured by the bound Zn(II) and the tetra-Cu(I)–thiolate cluster. As neither Ace1p nor Amt1p responds to exogenous Zn(II) via enhanced DNA binding or target gene transcription, it is likely that the Zn(II) ion plays a purely structural role in Cu-MRTFs and forms an independent module that correctly positions the minor-groove-binding domain with respect to the tetra-Cu-cluster DNA-binding domain.

Yeast genes encoding proteins that function in high affinity Cu(I) transport into the cell are transcriptionally regulated by Cu in the opposite direction from the detoxifying genes and by Cu concentrations that fall into the nutritional rather than toxic range. The transcription of *CTR1*, *CTR3* and *FRE1* is strongly repressed by elevated Cu concentrations and de-repressed by Cu starvation; these regulatory events are completely independent of the Ace1p Cu-MRTF [63–66]. Cu repression of these genes is rapid and exquisitely sensitive to, and specific for, Cu, with half-maximal repression of the *CTR3* promoter occurring at 2×10^{-11} M Cu. The observation that Ag(I) represses expression from the *CTR3* promoter with nearly identical efficacy (1.4×10^{-11} M) would suggest that, like the Ace1 and Amt1 proteins, the nutritional Cu sensor(s) that regulate Cu transporter gene expression recognize Cu(I) rather than Cu(II) [64].

The promoter elements in *CTR1*, *CTR3* and *FRE1* that are required for gene regulation as a function of changes in Cu levels are strongly conserved, with each of these promoters

having two copies of the Cu-responsive *cis*-acting element (CuRE) 5′-TTTGCTC-3′, in either inverted or tandem orientation [64]. The CuRE is strikingly similar in sequence to (but not functionally redundant with) the MREs bound by Ace1p and Amt1p, strongly suggesting that the *trans*-acting factors that directly bind to this element in the Cu-transporter gene promoters may have structural and/or biochemical properties in common with Ace1p and Amt1p. Indeed, a number of experiments have identified the yeast nuclear protein Mac1 as an essential component of the nutritional Cu-signaling pathway [63–66]. First, *mac1* deletion mutants of *S. cerevisiae* exhibit Cu-starvation phenotypes that are corrected by adding Cu. Second, Mac1p is essential for both Cu-mediated repression and Cu-starvation-induced activation of *CTR1*, *CTR3* and *FRE1* gene expression. Third, yeast strains that have a dominant allele of the *MAC1* gene, *MAC1*^{up1}, have a very high basal expression of these genes, are refractile to Cu, and therefore the high affinity Cu-transport genes are only poorly and transiently repressed in response to high Cu levels. Fourth, *in vivo* footprinting by probing dimethyl sulfate reactivity has shown that the CuREs of the *CTR3* promoter are occupied under conditions during which the gene is expressed, but not after Cu addition when *CTR3* expression is down-regulated. The CuREs are not occupied under either condition in a *mac1* deletion strain and are occupied, regardless of the Cu status of the cell, in a *MAC1*^{up1} strain. Fifth, Mac1p has been demonstrated to directly bind to DNA fragments containing wild-type, but not mutated, CuREs *in vitro*.

Mac1p has several structural features consistent with a possible function as a Cu(I)-responsive DNA-binding transcriptional regulatory protein (Figure 4). Like Ace1p and Amt1p, Mac1p has nearly 50% identity to the amino-terminal module which is thought to contact the minor groove of MREs and to bind Zn(II). Furthermore, Mac1p contains an abundance of cysteines, most notably two Cys–His repeats with the conserved arrangement of amino acids Cys–X–Cys–X_4–Cys–X–Cys–X_2–Cys–X_2–His (Figure 4). All of the *MAC1* dominant mutants thus far reported lie in either the central four cysteines or the first Cys–His repeat, suggesting that the two Cys–His repeats may not be functionally equivalent with respect to Cu(I) sensing. One potential mode of regulation by Mac1p is that the non-Cu metallated form of Mac1p may bind to CuREs *in vivo* and activate transcription of high affinity Cu(I) transport genes. The abundant cysteine residues may sense and bind intracellular Cu(I), inactivating the DNA-binding function of Mac1p, and therefore resulting in the inactivation of *CTR1*, *CTR3* and *FRE1* gene expression. A number of other mechanisms and models for Mac1p function in Cu(I)-dependent regulation of the Cu-transport genes are possible and await further genetic, biochemical and chemical studies.

Several mechanisms for Cu-dependent regulation occur at the post-transcriptional level. Elevated extracellular Cu levels, beyond the range in which the high affinity Cu-transporter function would be in demand, not only repress *CTR1* transcription but also trigger the degradation of Ctr1p at the plasma membrane [67]. This interesting mechanism provides yeast cells with an additional opportunity to prevent the accumulation of Cu to toxic levels. Degradation of Ctr1p occurs at the plasma membrane specifically in response to Cu and does not require internalization or vacuolar hydrolysis. Conversely, the lack of Cu can render other Cu-requiring proteins susceptible to degradation. For example, the metallated Ace1p, Amt1p or metallothionein proteins are more resistant to degradation than the corresponding apo-proteins. In some algae and cyanobacteria, a heme-containing c-type cytochrome can functionally replace plastocyanin under conditions of Cu starvation [4]. The ability of the unicellular green alga *Chlamydomonas reinhardtii* to reciprocally regulate the levels of plastocyanin and cytochrome c_6 protein in in response to changing environmental Cu levels provides a unique adaptive advantage to this organism. Interestingly, this reciprocal regulation occurs at different levels. The cytochrome c_6 gene (*cyc6*) is transcriptionally activated by Cu starvation, whereas the *petE* gene encoding plastocyanin is constitutively expressed. Under conditions of Cu starvation, the plastocyanin protein itself becomes unstable and is rapidly degraded by a protease activity present in Cu-deficient cells [68].

A recently discovered aspect of Cu-mediated regulation impinges on alterations in protein trafficking. In mammalian cells the expression of the *MNK* gene, encoding the putative Cu transporting P-type ATPase, is not regulated by Cu at the transcriptional or translational level. In studies of Cu-resistant variants of Chinese hamster ovary cells in which the *MNK* gene has been amplified, MNK localization is regulated by high or low Cu concentrations. In a low-Cu medium, the MNK protein is localized to the *trans*-Golgi network, where it is likely to participate in supplying Cu to the secretory network. When elevated Cu levels are present, however, the MNK protein is mobilized to the plasma membrane in a reversible, energy-dependent and protein-synthesis-independent fashion [69]. The observation that MNK trafficking from the Golgi to the plasma membrane is specific to Cu or Ag(I), suggests that Cu(I) is involved in this process, and predicts that perhaps the amino-terminal conserved metal-binding motifs of MNK may play a role in sensing the Cu that is required for trafficking, as well as in Cu compartmentalization into the secretory system. One hypothesis to explain the biological significance of this trafficking event is that, once at the plasma membrane, MNK may participate in Cu extrusion to protect cells from toxic levels of Cu.

In summary, Cu-dependent proteins carry out some of the most fundamental and specialized reactions in living organisms. Nature has designed a surprisingly broad range of

Cu-binding motifs to facilitate Cu binding in a manner that allows Cu to perform its function in each case or to allow Cu to be transported. Elucidation of the identities, structure and biological function of Cu-proteins has thus far involved fundamental areas of chemistry and biology: coordination chemistry, protein structure, genetics, cell biology, biochemistry and the clinical sciences. Recent discoveries of highly specialized proteins and enzymes exhibiting a Cu dependency, as well as novel Cu-dependent homeostatic control mechanisms, provide further evidence that we have much to discover in the area of the chemistry and biology of copper. The stage is now firmly set for cross-disciplinary studies towards a comprehensive understanding of the delicate orchestration of copper in biology.

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