1. Introduction

Plentiful and diverse hypotheses on the origin of life on Earth have included the archaic notion of spontaneous generation, the classic theory of a primordial soup, the exotic idea of panspermia or exogenesis, and modern hydrothermal vent theories. While emphasis usually focuses on the origin of organic compounds as the building blocks of life, modern theories include the contribution of metals in the process. Of particular interest for this review article is copper, a metal that was likely appropriated Fe$^{2+}$ and Mn$^{2+}$, as these were the most soluble and abundant. It was the utilization of Mn$^{2+}$ that led to the rise of photosynthetic cyanobacteria and the mass production of O$_2$.7 billion years ago (GYA) [4]. It is believed that for the first 200–300 million years O$_2$ produced by cyanobacteria in the upper ocean was consumed by the oxidation of Fe$^{2+}$ to Fe$^{3+}$, resulting in the precipitation of iron from the ocean surface as Fe(OH)$_3$. Continued oxygen generation stratified the ocean, with upper zones becoming oxic and the deeper waters remaining anoxic. The anoxic layer is also believed to have become sulfidic via continental weathering and the action of sulfate reducing bacteria [5]. The increased sulfide content would also contribute to the loss of oceanic iron via the formation of insoluble pyrite (FeS$_2$) [6], which, accompanied by continued oxygenation of the ocean and precipitation of iron as Fe(OH)$_3$, eventually resulted in the mass precipitation of iron from the ocean. Concurrently, copper was liberated from insoluble sulfide via oxidation of Cu(I) to the more soluble Cu(II).

The advent of O$_2$, the disappearance of soluble iron, and the concurrent solubilization of copper resulted in dramatic changes in the chemistry and biochemistry of early life [7]. Iron had become an essential element, acting as a cofactor in many enzymes. The precipitation of insoluble iron required a reinvention in the mechanisms used limited diversity of available free metal ions that developing life could utilize for metalloproteins. The first enzymes to incorporate metals likely appropriated Fe$^{2+}$ and Mn$^{2+}$, as these were the most soluble and abundant. It was the utilization of Mn$^{2+}$ that led to the rise of photosynthetic cyanobacteria and the mass production of O$_2$.7 billion years ago (GYA) [4]. It is believed that for the first 200–300 million years O$_2$ produced by cyanobacteria in the upper ocean was consumed by the oxidation of Fe$^{2+}$ to Fe$^{3+}$, resulting in the precipitation of iron from the ocean surface as Fe(OH)$_3$. Continued oxygen generation stratified the ocean, with upper zones becoming oxic and the deeper waters remaining anoxic. The anoxic layer is also believed to have become sulfidic via continental weathering and the action of sulfate reducing bacteria [5]. The increased sulfide content would also contribute to the loss of oceanic iron via the formation of insoluble pyrite (FeS$_2$) [6], which, accompanied by continued oxygenation of the ocean and precipitation of iron as Fe(OH)$_3$, eventually resulted in the mass precipitation of iron from the ocean. Concurrently, copper was liberated from insoluble sulfide via oxidation of Cu(I) to the more soluble Cu(II).

The advent of O$_2$, the disappearance of soluble iron, and the concurrent solubilization of copper resulted in dramatic changes in the chemistry and biochemistry of early life [7]. Iron had become an essential element, acting as a cofactor in many enzymes. The precipitation of insoluble iron required a reinvention in the mechanisms used

---

**ARTICLE INFO**

**Article history:**
Received 2 June 2011
Received in revised form 25 October 2011
Accepted 15 November 2011
Available online 3 December 2011

**Keywords:**
Copper
Cuproprotein
Copper protein
Metal homeostasis

**ABSTRACT**

Biological copper is coordinated predominantly by just three ligand types: the side chains of histidine, cysteine, and methionine, with of course some exceptions. The arrangement of these components, however, is fascinating. The diversity provided by just these three ligands provides choices of nitrogen vs. sulfur, neutral vs. charged, hydrophilic vs. hydrophobic, susceptibility to oxidation, and degree of pH-sensitivity. In this review we examine how the total number of ligands, their spatial arrangement and solvent accessibility, the various combinations of imidazole, thiolate, and thioether donors, all work together to provide binding sites that either enable copper to carry out a function, or safely transport it in a way that prevents toxic reactivity. We separate copper proteins into two broad classes, those that utilize the metal as a cofactor, or those that are essentially independent of the metal. Enzymes and proteins that utilize copper as a cofactor use high affinity sites of high coordination numbers of 4–5 that prevent loss of the metal during redox cycling. Copper trafficking proteins, on the other hand, promote metal transfer either by having low affinity binding sites with moderate coordination number ~4, or by having lower coordinate binding sites of 2–3 ligands that bind with high affinity. Both strategies retain the metal but allow transfer under appropriate conditions. Analysis of studies from our own lab on model peptides, combined with those from other labs, raises an interesting hypothesis that various methionine/histidine/cysteine combinations provide organisms with dynamic, multifunctional domains on copper trafficking proteins that facilitate copper transfer under different extracellular, subcellular, and tissue-specific scenarios of pH, redox environment, and presence of other copper carriers or target proteins.

© 2011 Elsevier Inc. All rights reserved.

---

**Focused review**

Coordination chemistry of copper proteins: How nature handles a toxic cargo for essential function

Jeffrey T. Rubino, Katherine J. Franz*

Department of Chemistry, Duke University, Durham, NC 27708-0346, United States

---

*Corresponding author. Tel.: +1 919 660 1314; fax: +1 919 660 1605.
E-mail address: katherine.franz@duke.edu (K.J. Franz).

0162-0134/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2011.11.024
to acquire it. Early life adjusted by excreting organic acids and strong iron chelators ( siderophores) to solubilize the metal, and developed sophisticated methods by which to then obtain it [1]. Organisms also evolved methods to sequester Fe$^{3+}$ for later use, most notably via ferritin, the ubiquitous iron storage protein [8].

The rise of O$_2$ altered the redox chemistry of the environment. In the absence of O$_2$, life had adapted to reaction systems in the lower portion of the redox potential spectrum, limited by the H$_2$S/S$^-$ and H$_2$/H$^+$ potentials ranging 0.0 to $-0.4$ V at a pH of 7 [9]. The presence of O$_2$ increased the upper limit of the range to 0.8 V, leading to oxidation and degradation of organic compounds and cellular components vital for life. Soluble copper also proved hazardous to early life as it can potentially replace essential metal cofactors, including Zn [10] and Fe [11], in proteins, and catalyze the formation of reactive oxygen species (ROS) that have been associated with DNA/RNA lesions, protein oxidation, and lipid peroxidation [12-16]. Oxygen would eventually transform from poison to a necessity as the final electron acceptor to produce ATP in aerobic respiration. Life would also evolve uses for copper, thus transforming a potential poison into a useful and required cofactor. As a result, life evolved highly sophisticated networks that would allow organisms to acquire, transport, sequester, and export copper to maintain requisite amounts while preventing accumulation of toxic levels. In this review, we draw a distinction between two very broad classes of copper proteins: (i) proteins that utilize copper as a cofactor to carry out a specific function, and (ii) proteins that transport copper as cargo. By exploring the metal–ligand coordination environments of these broad groups of proteins, we hope to elucidate the chemical principles Nature uses to handle a toxic cargo needed for essential function.

### 1.1. Metal–ligand coordination chemistry: copper aspects

Before examining different types of copper centers found in cuproproteins, it is first important to understand the principles of metal–ligand coordination chemistry as they relate to the composition and geometries we should expect to find. We will soon observe that protein copper binding sites are dominated by histidine, cysteine, and methionine residues, shown in Fig. 1. This occurrence is in agreement with the Hard and Soft Acids and Bases (HSAB) principle [17,18].

Applying HSAB to the metal of interest, we see that Cu(I) is a soft acid while Cu(II) is borderline, thus, we would expect copper binding sites to be dominated by amino acids containing side chains with soft or borderline ligands. Amino acids with nitrogen and sulfur donor atoms, histidine and cysteine and methionine, respectively, would therefore be preferred over amino acids with hydroxyl, carboxyl, or primary amine side chains, as found in serine and tyrosine, aspartate and glutamate, and asparagine, glutamine, and arginine, respectively.

Metals exhibit preferences in binding geometry based on the number of valence d electrons and the number and type of coordinating ligands, as explained through the principles of ligand field theory (LFT) [19]. Based on LFT, differences in geometric preferences for Cu(I) and Cu(II) are expected. Cu(I) is a d$^9$ system and therefore does not have the same pH dependence as histidine and cysteine and is less prone to oxidation than cysteine (although it can be oxidized under some conditions to methionine sulfoxide and methionine sulfoxide). The methionine side chain is also considerably more hydrophobic than the other two residues, a property that can influence solvent accessibility and protein–protein contacts.

Another important feature of the ligand set (both its composition and its geometric arrangement) is its influence on the reduction
the explosion of photosynthetic bacteria likely affected the evolution of vis and EPR spectroscopy, and X-ray crystallography.

2. Proteins and enzymes that use copper for function

The first copper proteins to consider are those that evolved to utilize copper as a cofactor to perform a biological function. As a result of the environment, many of these proteins evolved functions related to oxygen. Most members of this group bind copper with high affinity in high-coordinate binding sites to prevent loss of the metal during redox cycling [22]. For copper, high coordination binding sites refer to coordination numbers of 4–5. These binding sites have been well defined and characterized by a variety of techniques, including UV–vis and EPR spectroscopy, and X-ray crystallography. Figs. 2–6 depict the composition, relative orientation, and approximate geometry for the inner coordination spheres of several of these cuproproteins for which crystal structures are known.

2.1. The emergence and function of copper as a cofactor

The oxygen rich and iron deficient environment that accompanied the explosion of photosynthetic bacteria likely affected the evolution of the first copper proteins [7,23]. The presence of oxygen, and the resulting expansion of the range of available redox potentials into positive values, made new energy sources and new chemistry possible. Where iron was the early cofactor of choice to catalyze redox reactions, organisms were hard-pressed to obtain it[1]. The bioenergetically costly strategies of iron complexation and/or reduction left abundant and soluble copper to serve as a cofactor in enzymes filling these new niches.

Evidence for this change in metal preference as a response to the rise of oxygen can be seen when comparing the reduction potentials of copper-containing proteins to iron-containing proteins, and also comparing iron-containing proteins that evolved before and after utilization of oxygen (Table 2) [7]. Copper proteins have positive reduction potentials, while only iron proteins that have an oxygen-related function have positive reduction potentials. Other evidence to support this theory can be found in bioinformatic analyses of the metalloproteome of aerobic vs. anaerobic organisms. Almost all aerobic organisms contain copper proteins compared to very few anaerobic organisms that have functionalized the metal [24,25]. A phylogenomic analysis of metal binding protein structures suggests that the protein folds for binding Fe, Mn, and Mo evolved prior to the global

![Fig. 2. Examples of type I copper centers: plastocyanin, umecyanin, azurin.](image-url)
oxidation event, while the folds for Cu and Zn developed after [26].
The study also suggests that the percentage of metal binding sites
within a proteome remains constant over time, and thus inclusion
of a new metal (i.e. copper) is usually at the expense of another (i.e.
iron).

Primary functions of copper proteins include electron transfer,
catalyzing redox reactions of various biological substrates, and dioxy-
gen transport [23]. Select examples of copper proteins and their func-
tions are summarized in Table 3. Considering the previously discussed
hypothesis involving the rise of copper proteins, it is not surprising to
find that many of them have oxygen-related functions.

2.2. Copper center classifications and example cuproproteins

Proteins utilizing copper as a cofactor are often classified into
groups based on ligand composition and geometry of the metal center.

These copper centers exhibit very similar characteristics, including UV–
vis and EPR signals, and reduction potentials, regardless of the protein
within which they are found. The different types of copper centers, in-
cluding the number of copper atoms and metal center geometry, are
listed in Table 4, along with example proteins and their respective cop-
per coordination environments.

The type I (T1Cu) group of copper proteins is probably the most
studied and characterized. Most members of this group are electron
transfer proteins, and are also known as the cupredoxins. All mem-
bers of this group contain 1 copper atom coordinated by 2 histidine
imidazole nitrogens and 1 cysteine thiolate sulfur in a trigonal planar
fashion, with further classification depending on the number and
type of axial ligands. Class I T1Cu centers have a methionine thioether
sulfur weakly bound in the axial position, while those in class II have a
non-methionine axial ligand, both resulting in a distorted tetrahedral
geometry. Class III T1Cu centers have 2 axial ligands, one of which is

\[
\text{Hemocyanin} \\
\text{Type III} \\
[Cu(N_\text{His})_3]_2 \quad \leftrightarrow \quad \mu \text{O}_2[Cu(N_\text{His})_3]_2
\]

Fig. 4. Example of a type III copper center: hemocyanin. Deoxygenated hemocyanin is shown on the left, and the oxygenated form on the right. Upon oxygen binding, the copper atoms are drawn closer together, switching from a trigonal planar geometry to that of a trigonal bipyramidal geometry, characteristic of type II copper centers.

![Diagram of Hemocyanin]

![Diagram of Cytochrome c Oxidase]

Fig. 5. Example of CuA and CuB copper centers: cytochrome c oxidase.
methionine, and the other is a glycine carbonyl oxygen, resulting in a distorted trigonal bipyramidal geometry. Examples of Class I, II, and III T1Cu centers from plastocyanin [39], umecyanin [40], and azurin [41], respectively, are shown in Fig. 2. All T1Cu proteins have positive reduction potentials greater than 0.25 V. They also have a strong absorbance around 600 nm as a result of the cysteine thiolate sulfur-to-copper charge transfer band, giving rise to yet another name for this group, the blue copper proteins. T1Cu proteins also exhibit small hyperfine splitting in the parallel region of EPR spectra [42].

Type II copper centers (T2Cu) are often found in the oxidoreductases. The metal center is coordinated predominately by histidine residues in a distorted square planar geometry; oxygen-containing ligands are also common. Cu, Zn superoxide dismutase (Cu, Zn SOD) is a well-known member of this group. The Cu, Zn SOD T2Cu center is shown in Fig. 3, water has been placed in the substrate binding axial position [43]. T2Cu centers lack distinctive features in absorbance spectra as they lack sulfur ligands. They are, however, EPR active, usually exhibiting axial symmetry and hyperfine splitting [44]. The copper-binding site of the amyloid precursor protein (APP) has also been described as a T2Cu center. Copper here is thought to be coordinated by two histidine residues, a tyrosine residue, and two water molecules in a distorted square pyramidal geometry, which gives rise to T2Cu EPR and EXAFS spectra [45,46]. The function of the APP is unknown; however, as the type of center suggests, copper is redox active.

Type III copper centers (T3Cu) are binuclear copper centers, with each copper bound by 3 histidines. They are found in some oxidases, and the arthropod oxygen transport protein, hemocyanin. In the absence of dioxygen, each copper atom in the T3Cu center is in a trigonal planar geometry [47]. Upon dioxygen binding, the copper atoms are drawn closer together, and take on a trigonal bipyramidal geometry [48]. This process is shown in Fig. 4. UV–vis spectroscopy of the oxygen-loaded hemocyanin shows a strong absorbance between 350 and 580 nm. The protein is EPR silent due to the antiferromagnetic coupling of the two S=1/2 Cu(II) atoms, resulting from the bridging O2 ligand [49].

Copper A and B centers, CuA and CuB respectively, are both found in cytochrome c oxidase (CcO), a large multidomain protein that is the last enzyme in the electron transport chain (Fig. 5) [50]. The mixed valence CuA center is located in the Cox2 domain and contains 2 copper atoms bridged by 2 cysteine residues, both in tetrahedral geometry. The first is also coordinated by a histidine and a methionine, while the second is coordinated by a histidine and the carbonyl oxygen of a glutamate residue. The CuA site is believed to be the cytochrome c electron acceptor, which then donates the electron to the internal heme. In the CuB site, located in the Cox1 domain,
copper is bound by 3 histidine residues in a trigonal pyramidal geometry. The open face of the CuB is believed to be part of an oxygen binding site involved in electron transfer from the heme.

The last type of copper center, and the most recently discovered, is the copper Z center (CuZ) found in a N₂O reductase. The CuZ center contains 4 copper atoms arranged in a distorted tetrahedron coordinated by 7 histidines and bridged by an inorganic sulfur ion (Fig. 6) [51]. The fourth copper atom, ligated by only 1 histidine and an unknown oxygen species, is believed to be the location of substrate binding. Copper centers are not mutually exclusive, N₂O oxidase also contains a CuA site. Laccase and ascorbate oxidase each contain a T¹Cu center, and a trinuclear T²Cu/T³Cu center [52,53]. In both cases the inner coordination sphere around copper are shown in Figs. 7–10 for select examples. Dashed lines between a donor atom and Cu are used to indicate that the structure has not been determined by X-ray crystallography, although other data were used to assess the composition of the site.

### 3. Copper trafficking proteins

Copper trafficking proteins are those that transport copper as cargo. We include in this categorization integral membrane proteins that translocate copper across cell and organelle membranes, intracellular copper chaperones responsible for shuttling the cofactor to respective cuproenzymes, resistance proteins utilized to prevent accumulation of toxic levels of copper, and the copper binding transcription factors and other regulatory proteins that affect expression of other proteins that have been implicated in copper regulation (or misregulation), although their roles are still unclear. Notable examples include the amyloid precursor protein (APP), prion proteins, and synucleins, which all are associated with neurodegenerative diseases. While these proteins will not be discussed further here, their copper chemistry has been extensively reviewed elsewhere [54].

With the possible exception of the transcription factors/regulatory proteins, copper trafficking proteins contain binding sites with either low affinity or low-coordinate flexible geometries that enable transfer to the recipient protein or enzyme. The chemical structures of the inner coordination sphere around copper are shown in Figs. 7–10 for select examples. Dashed lines between a donor atom and Cu are used to indicate that the structure has not been determined by X-ray crystallography, although other data were used to assess the composition of the site.

### 3.1. Integral membrane copper transport proteins

There are currently two families of integral membrane proteins validated as copper transporters, the P₁β-type ATPases and Ctr proteins. While Ctr proteins are found exclusively in eukaryotes, copper ATPases are ubiquitous in all three domains of life. In eukaryotes, the ATPases translocate copper into the lumen of the Golgi, where it is then incorporated into cuproenzymes destined for the plasma membrane or for secretion out of the cell [55]. The importance of this pathway to human health is highlighted by the fact that mutations in ATP7A and ATP7B lead to Menkes syndrome and Wilson’s disease, respectively. Archaeal and bacterial ATPases are typically associated with a detoxification role to rid the cell of excess copper and allow the organism to survive under high copper conditions [56]. Some pathogenic bacteria, for example, rely on Cu ATPases for infection and virulence in response to an elevated copper concentration imposed by the host immune response [57]. Another function of prokaryotic Cu ATPases is to facilitate cuproprotein assembly [57]. A list of example functions of copper transporting ATPases is shown in Table 5. Whereas Cu ATPases almost exclusively efflux Cu out of the cytoplasm [57], Ctr proteins import Cu into the cell or vesicle. Copper

---

**Table 4**

Characteristics and examples of different copper centers.

<table>
<thead>
<tr>
<th>Center</th>
<th># Cu</th>
<th>Geometry</th>
<th>Example</th>
<th>Coordination environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>1</td>
<td>Tetrahedral</td>
<td>Plastocyanin</td>
<td>Cu(N₁₅H₂O)S₄CyS₄Met</td>
</tr>
<tr>
<td>Class I</td>
<td>1</td>
<td>Tetrahedral</td>
<td>Umecyanin</td>
<td>Cu(N₁₅H₂O)S₄Cy₄O₅</td>
</tr>
<tr>
<td>Class II</td>
<td>1</td>
<td>Trigonal bipyramidal</td>
<td>Azurin</td>
<td>Cu(N₁₅H₂O)S₄Cy₅O₅</td>
</tr>
<tr>
<td>Type II</td>
<td>1</td>
<td>Square pyramidal</td>
<td>Cu, Zn SOD</td>
<td>Cu(N₁₅H₂O)[H₂O]</td>
</tr>
<tr>
<td>Type III</td>
<td>2</td>
<td>Trigonal bipyramidal</td>
<td>Hemoxygenin</td>
<td>μ₂[Cu(N₁₅H₂O)]</td>
</tr>
<tr>
<td>Cu₄</td>
<td>2</td>
<td>Tetrahedral</td>
<td>Cytochrome c oxidase</td>
<td>μ[2Cu(N₁₅H₂O)²]</td>
</tr>
<tr>
<td>Cu₅</td>
<td>1</td>
<td>Trigonal pyramidal</td>
<td>Cytochrome c oxidase</td>
<td>Cu(N₁₅H₂O)</td>
</tr>
<tr>
<td>Cu₆</td>
<td>4</td>
<td>Tetrahedron</td>
<td>N₂O reductase</td>
<td>μSi²⁻{4Cu(N₁₅H₂O)O(?)}</td>
</tr>
</tbody>
</table>

* Copper centers are often described as having distorted versions of these geometries

---

**Fig. 7.** Examples of copper binding sites in ATPases and chaperones.

---
ATPases and Ctr proteins have significantly different mechanisms of transport, structures, and copper binding sites.

P1B-type ATPases catalyze the dephosphorylation of ATP to ADP and inorganic phosphate, utilizing the energy released to transport copper across a membrane. Copper ATPases are comprised of multiple domains, including a pore forming transmembrane domain comprised of 8 α-helices, cytosolic nucleotide binding and phosphorylation domains, and a variable number of soluble protein domains located at the N-terminus, each containing a copper-binding motif [63,64]. The number of these domains appears to increase with increasing complexity of the organism. NMR analyses of ATPases reveal that they bind copper in a CXXC binding motif with near linear S–Cu–S bond angles ranging 120–170° (Fig. 7) [65–67]. The binding site is presumed to be flexible and capable of accommodating a diversity of geometries, particularly when the metal is transferred to and from the ATPase, resulting in the observed deviations from a pure linear geometry. The transmembrane domain contains at least 2 copper binding sites. A strictly conserved CPC or CPH (also SPC and possibly others [64]) motif located in 6th transmembrane helix is required for transport function [68]. The binding sites are formed by this motif and the contribution of a strictly conserved NXXXXXYN and highly conserved MXXSS/TSS motifs located in the 7th and 8th helices, respectively. X-ray spectroscopy and metal-binding mutagenesis studies suggest the existence of two trigonal planar binding sites, one comprised of 2 cysteine residues and a tyrosine residue, and the second comprised of cysteine, methionine, and asparagine residues [69,70].

Ctr proteins are ubiquitously expressed among all eukaryotes, and have been identified in yeast, insects, mice, humans, and various plants [71–76]. Ctr proteins do not utilize ATP as an energy source to transport copper across membranes [77]. Instead, the transporter takes advantage of the favorable concentration gradient as intracellular copper is effectively sequestered. Ctr proteins are symmetrical homotrimers that form a pore at the interface of the 3 α-helix-containing subunits [78–80]. The N-terminal region of the protein located on the cell surface contains methionine residues (Mets motifs) implicated in copper transport (Fig. 8) [81]. Some organisms, including mammals and the green algae *Chlamydomonas* [76], utilize histidine and/or cysteine in concert with methionine at the cell surface. Work in our lab has identified histidine-containing copper binding motifs on the extracellular domain of mammalian Ctr1, including a Cu(II) specific H$_2$N-XXH amino terminal copper/nickel (ATCUN) square planar binding site, and Cu(I) bis-histidine (HH) binding sites (Fig. 8) [82]. Methionine motifs are also important for shuttling copper down the pore [80,81]. The C-terminal region of the protein located in the cytoplasm lacks methionine but contains histidine and cysteine motifs that are believed to transfer copper to chaperones [80,83]. Yeast Ctr1 binds 4 equivalents of Cu(I) with 6 cysteine residues, reminiscent of, albeit with fewer atoms than, metallothionein, and identical to the binding
site in Ace1 regulatory protein, as revealed by EXAFS [83]. EXAFS studies also revealed that human Ctr1 binds Cu(I) in a cysteine or histidine rich environment via an HCH motif, however, the biologically relevant coordination environment is unknown [80]. *Chlamydomonas* Ctr1 presumably binds Cu(I) in a similar environment via a CXCXH motif [76].

### 3.2. Copper chaperones

Cells utilize chaperone proteins to safely ferry copper to the desired location, while preventing redox cycling and the resulting production of damaging reactive oxygen species. Known copper chaperones can be divided into 3 groups, those that transport the metal to ATPases, those that deliver the cofactor into Cu, Zn SOD (SOD1), and those that shuttle copper to the mitochondria and/or assist with incorporation into Co [84]. Copper chaperones are located in the periplasm of gram-negative bacteria, and the cytosol and the inner membrane space (IMS) of the mitochondria of eukaryotes. Most chaperones utilize the CXXC motif; some also appear to incorporate histidine into the binding site (Fig. 7). Examples of copper chaperones, including recipient proteins/enzymes, locations, and binding site compositions, are listed in Table 6.

Chaperone proteins that deliver copper to ATPases are often referred to as Atx1-like copper chaperones, named after the yeast version [91]. They are usually around 70 amino acids, and contain the CXXC binding motif. NMR analyses have revealed that the Atx1-like chaperones do not coordinate copper in a pure linear geometry. The S–Cu–S bond angles observed in Atx1 and CopZ range 115–120°, which suggests a geometry closer to that of a distorted tetrahedron [117,118]. While EXAFS data suggest the presence of a possible additional sulfur donor, it is unclear if the bond angles determined by NMR are just a reflection of the flexibility of the binding motif, or if there is an unknown exogenous third ligand [119]. It is possible that EXAFS is detecting a coordination environment similar to the Cu(I)-bridged dimer observed in the crystal structure of the human version of the chaperone, Hah1/Atx1. Here, two Hah1 molecules bound Cu(I) via three cysteine residues in a distorted tetrahedral geometry [89]. However, the NMR solution structure and EXAFS studies suggest the protein exists as a monomer where Cu(I) is bound by 2 cysteines in a near linear geometry with a S–Cu–S bond angle ranging 135–185° [90,120]. ScAtx1 and *B. subtilis* CopZ contain a histidine near the binding site, however it is only involved in copper binding in the ScAtx1 version [94,95].

Most eukaryotes utilize CCS to deliver copper to Cu,Zn SOD located primarily in the cytosol, but also the IMS [121,122]. Prokaryotes do not have homologues for CCS, as Cu, Zn SOD in gram-negative bacteria is located in the periplasm. Structural determination of the human and yeast version of the protein are still incomplete. They appear to be large, around 27 kDa, proteins comprised of 3 domains, the first of which has considerably homology to Atx1 and also contains the CXXC motif [101]. The second domain contains similar structural elements to Cu, Zn SOD, however lacks a copper-binding site. The third, unstructured domain contains a conserved CXC motif that is known to be critical for activity [123]. Transfer of copper from CCS to Cu, Zn SOD likely

---

**Table 5**

Examples and functions of copper P1B-type ATPases.

<table>
<thead>
<tr>
<th>Organism</th>
<th>ATPase</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Eukaryote</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Ccc2</td>
<td>Recruit copper to the Golgi apparatus for incorporation into cuproenzymes.</td>
<td>[58]</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>MNK (ATP7A)</td>
<td>Recruit copper to the Golgi apparatus for incorporation into cuproenzymes.</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>WLN (ATP7B)</td>
<td>Under high copper, these proteins can redistribute to the cell membrane or endocytic vesicles to allow export or storage of excess copper. WLN expressed in non-liver tissue, WLN expressed in liver.</td>
<td>[55]</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>HMA5–8</td>
<td>Copper transport into the chloroplast and the post-Golgi compartment, and copper detoxification in the roots.</td>
<td>[59]</td>
</tr>
<tr>
<td><strong>Prokaryote</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>CopA</td>
<td>Exports copper from cytosol to periplasm</td>
<td>[60]</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. PCC 6803</td>
<td>CtaA</td>
<td>Involved in copper tolerance as well as assembly of cuproproteins in the photosynthetic pathway. CtaA located on plasma membrane, while PacS is on the thylakoid membrane.</td>
<td>[57,61]</td>
</tr>
<tr>
<td></td>
<td>PacS</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. acidarmanus</em></td>
<td>CopB</td>
<td>Assists in copper tolerance that allows growth in levels of copper that reach 20 g/L.</td>
<td>[62]</td>
</tr>
</tbody>
</table>

---

**Fig. 10.** Examples of copper binding sites in Cop/PcoC.
involves the formation of a transient heterodimer [98,100,122,124,125]. CCS-independent loading of copper into Cu, Zn SOD has also been reported [121,126–128].

The exact mechanism of how copper is transported to the mitochondria and then incorporated into CcO is still being elucidated. Cox17 was initially believed to shuttle copper from the cytosol to the mitochondrial IMS, however Cox17 tethered to the inner mitochondrial membrane is fully functional, and mitochondrial copper levels are independent of Cox17 [129,130]. Recent evidence suggests that there may be a low molecular weight copper ligand that is responsible for shuttling copper to the mitochondria [130,131].

Regardless of mechanism, a great deal is still known about the copper chaperones involved in inserting copper into CcO once inside mitochondria. Cox17 is a small, 64 amino acid, cysteine rich protein where, although polycopper clusters have been reported [132], the biologically relevant binding site appears to be a CC motif that coordinates the metal in a near linear geometry, with a S-Cu-S bond angle of 130° [106–108]. Cox17 has been shown to transfer copper to Cox11 and the Sco proteins [105,106,109], which then insert copper into the Cox1 and Sco2 domains of CcO, respectively [103,111–113,115,116]. Cox11 is tethered to the inner mitochondrial membrane by an N-terminal linker, the 164 amino acid protein contains a CXXC copper-binding motif. NMR and EXAFS analysis of a soluble anchorless construct has suggested the formation of a dimeric protein with two cysteine residues bridging two Cu(I) ions [102]. Both Sco1 and Sco2 are large proteins, around 30 kDa, and like Cox11, are also anchored to the inner mitochondrial membrane. Sco1 and Sco2 both bind Cu(I) in a near trigonal planar geometry utilizing a CXXXC motif, and a distant histidine residue [113–115]. It has been suggested that Sco1 and Sco2 also contain a Cu(II) binding site, based on hyperfine splitting in the EPR consistent with a square planar or square pyramidal T2Cu center [133]. Only two residues have been identified as potential Cu(II) coordinating ligands: a conserved histidine and aspartate.

3.3. Copper resistance proteins

Protein networks involved in copper resistance are predominantly found in prokaryotes, particularly gram-negative bacteria [134]. Bacteria remove excess copper from the cytosol by utilizing the ATPase CopA. While this effectively removes the poisonous metal from a gram-positive bacterium, gram-negative bacteria still need to manage copper that enters the periplasm. Two copper handling systems have been well characterized that accomplish this: Cus, a proton driven CBA transport system, and the plasmid-encoded pco or cop determinant.

The Cus pathway is a CBA export system whereby three proteins, CusA, an inner membrane protein of the resistance nodule division (RND), CusB, a membrane fusion protein, and CusC, an outer membrane factor, form a tripartite transport complex that spans the width of the periplasm [135–137]. CusA has been characterized by X-ray crystallography, and shown to bind Cu(I) and Ag(I) in a 3 coordinate methionine-only binding site for Cu(I) and Ag(I) that has been characterized by biochemical and EXAFS studies [139]. A crystal structure of CusC does not reveal any obvious metal-binding features, suggesting that the Cu(I)/Ag(I) specificity of the Cus system likely comes from the CusA and CusB components [140]. Unlike other similar resistance pathways in gram-negative bacteria utilizing the CBA complex, the Cus determinant employs a novel protein, CusF, that functions as a copper chaperone [141]. CusF is believed to deliver Cu(I) and Ag(I) to CusB [142], inducing a conformational change in CusB [139], and triggering metal efflux via an unknown mechanism. The process may be like a ‘funnel’ where metals bound to CusB are then transferred to CusA and then ejected out of the cell, or the CusF-to-CusB metal transfer may act as a ‘switch’ for the CBA complex, allowing efflux of free periplasmic metals [143]. Energy for the process is believed to be supplied by CusA via proton-substrate antiport [144,145]. A genomic analysis has revealed that about 3% of genes encoding CusF-like proteins may be expressed in the absence of CusCBA or other putative copper resistance systems [146]. In these cases the protein may only sequester the metal. CusF has been well defined structurally, and binds Cu(I) and Ag(I) with 2 methionines, 1 histidine and a tryptophan that provides a novel cation-π interaction with the bond metal [Fig. 9] [147,148].

The mechanisms of plasmid-encoded Pco and Cop copper resistance pathways are less defined. These resistance systems were first discovered in bacteria able to survive in environments with such high concentrations of copper that it would compromise the normal, chromosomally encoded, copper homeostatic system [149,150]. These plasmid-based resistance networks are comprised of 4 to 5 copper proteins, the best studied include the E. coli PcoABCDE and P. syringae.

---

Table 6

Examples of copper chaperones.

<table>
<thead>
<tr>
<th>Chaperone</th>
<th>Organism</th>
<th>Recipient</th>
<th>Location</th>
<th>Binding site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase related</td>
<td>Halt1/Atx1</td>
<td>H. sapiens</td>
<td>MNK/WLN</td>
<td>Cytosol</td>
<td>CXXC</td>
</tr>
<tr>
<td></td>
<td>Atx1</td>
<td>S. cerevisiae</td>
<td>CcA2</td>
<td>Cytosol</td>
<td>CXXC</td>
</tr>
<tr>
<td></td>
<td>ScaAtx1</td>
<td>Synechocystis sp. PCC 6803</td>
<td>PacS</td>
<td>Cytosol</td>
<td>CXXC + H</td>
</tr>
<tr>
<td></td>
<td>CopZ</td>
<td>E. hirae</td>
<td>CopA</td>
<td>Cytosol</td>
<td>CXXC</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD related</td>
<td>hCCS</td>
<td>H. sapiens</td>
<td>hSOD1</td>
<td>Cytosol</td>
<td>CXXC, CXC</td>
</tr>
<tr>
<td></td>
<td>yCCS</td>
<td>S. cerevisiae</td>
<td>ySOD1</td>
<td>Cytosol</td>
<td>CXXC, CXC</td>
</tr>
<tr>
<td>CoC related</td>
<td>Sco1</td>
<td>H. sapiens</td>
<td>Cox1</td>
<td>IMS</td>
<td>CXC</td>
</tr>
<tr>
<td></td>
<td>Sco2</td>
<td>S. cerevisiae</td>
<td>Sco1, Sco2, Cox11</td>
<td>Cytosol/IMS</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td>DR1885</td>
<td>D. radiodurans</td>
<td>Sco1</td>
<td>periplasm</td>
<td>MX20MX21M</td>
</tr>
<tr>
<td></td>
<td>Sco1</td>
<td>H. sapiens</td>
<td>Sco2</td>
<td>IMS</td>
<td>CXXX + H</td>
</tr>
<tr>
<td></td>
<td>Sco2</td>
<td>S. cerevisiae</td>
<td>Cox2</td>
<td>IMS</td>
<td>CXXXC + H</td>
</tr>
</tbody>
</table>

---

CopABC/D, and 2 regulatory proteins, PcoRS and CopRS. The proposed functions and locations of the Pco and Cop proteins are listed in Table 7 [151]. Pco/CopA\(^1\) exhibit oxidase activity [152]. Pco/CopC and Pco/CopD are required for full copper resistance. Pco/CopM may recruit copper to Pco/CopD where the metal is transported into the cytosol for incorporation into apo-Pco/CopA, prior to translocation of the holooenzyme into the periplasm [151,153,154]. PcoE is a known copper binding protein, and Pco/CopRS regulate expression of the pathway [155,156]. The only structurally characterized proteins from these pathways are PcoC and CopC, both of which appear to bind Cu(I) and Cu(II) in separate sites. EXAFS studies of \(E.\) coli PcoC suggest that Cu(I) is bound trigonally by 2 methionines and 1 histidine (Fig. 10) [157]. Cu(II) coordination is less defined, with EXAFS suggesting a four-coordinate site with at least 1 histidine ligand, 2 other nitrogen donors and 1 oxygen donor [152]. Structural analyses of \(P.\) syringae CopC produced somewhat conflicting results for the Cu(I) binding site. NMR and EXAFS suggest that Cu(I) is most likely 4 coordinate, bound by 3 methionine and one histidine residues, in a tetrahedral geometry, while the crystal structure suggests a methionine-only binding site (Fig. 10) [158–161]. The Cu(II) binding site was less ambiguous, with all techniques suggesting coordination by at least 2 histidine residues and two other N/O donors, one of which could possibly be a third histidine.

Eukaryotes employ different strategies to deal with excess cellular copper, one example being sequestration via metallothionein. Many studies have shown that the metallothionein plays a role in copper detoxification [162–165]. Other studies suggest that it may store the metal until it is required for insertion into apo-copper chaperones [166,167] and apo-copper enzymes [163,164]. These theories are certainly not mutually exclusive. Metallothionein is a small protein, 53 amino acids, containing 12 cysteine residues, and has been observed to bind between 6 and 8 equivalents of copper [168,169]. EXAFS analyses of the full-length protein and truncated construct have revealed that only 10 of the 12 cysteine residues bind copper [170,171]. There is a long history of attempts to determine the structure of copper loaded metallothionein. Crystallization of the full-length protein was never successful, and while NMR structures have been determined with increasing accuracy, information regarding the copper cluster could not be inferred [170,172–175]. Crystallization of the previously mentioned truncated construct was successful and has revealed that 6 of the copper are coordinated trigonally by cysteines, with the remaining 2 coordinated diagonally [176]. The diagonal coordination may play an important role in copper loading and unloading of metallothionein. The diagonal and trigonal mixed geometry is supported by previous EXAFS studies [171]. Other eukaryotic responses to high levels of copper are still being elucidated, some of which are discussed in the following section.

### 3.4. Transcription factors and regulatory proteins

Transcription factors for copper trafficking proteins regulate protein expression via interactions with promoter regions of DNA. Copper binding in these transcription factors is accompanied by structural rearrangements that either enhance or suppress the protein–DNA interaction, thereby either activating or blocking expression of the encoded copper trafficking proteins. In contrast to transcription factors, here we use the term regulatory proteins to describe proteins that affect cellular copper levels, but not by direct protein–DNA interactions nor by being directly involved in the transfer of the metal, as seen in the chaperones. These groups of proteins are some of the least defined, especially in regards to protein structure and coordination chemistry, and currently an area that certainly requires more scrutiny. Some of the best-studied members of these groups, and their functions, are listed in Table 8.

Yeasts possess 2 copper dependent nuclear transcription factors, Mac1 and Ace1 [185]. In Cu-deficient cells, Mac1 activates the expression of Ctr1, Ctr3, and the metalloreductase Fre1 [177]. High levels of copper inactivate Mac1 and activate Ace1, which subsequently promotes the expression of the metallothioneins CUP1 and CRSS, and Cu, Zn SOD [178,179]. Both proteins bind 4 equivalents of Cu(I), with 6 ligands in distorted tetrahedral cage; Ace1 utilizes 6 cysteine residues while Mac1 utilizes 5 cysteines and 1 histidine residue, with each Cu(I) coordinated by 3 ligands. Both coordination environments result in identical EXAFS spectra and are nearly identical to the EXAFS spectrum of the copper-bound, cysteine rich, cystosolic domain of yCtr1 [83,186].

Two of the most well-known bacterial transcription factors include CsoR and CueR. CsoR is widely distributed across many species of bacteria and has been proposed to be the primary prokaryotic copper sensor in bacteria lacking CueR [181]. CueR represses transcription of the copZ operon, in a process that is inhibited by high levels of copper [180]. A crystal structure and XAS experiments reveal that CsoR binds two equivalents of Cu(I) in a homodimer, wherein each Cu center is coordinated in a trigonal environment by a cysteine residue from one subunit of the homodimer, and an additional cysteine and histidine residue from the other subunit [181]. CueR behaves similarly, repressing transcription of the copA operon in a copper inhibited process. The CueR crystal structure from \(E.\) coli suggests that the protein binds Cu(I) in a nearly linear geometry via 2 cysteine residues [182].

There are at least 2 cytosolic regulatory proteins identified to date in mammals that are activated by increased levels of copper: XIAP, the X-linked apoptosis inhibitor, and COMMD1, a protein involved in many regulatory pathways [184,187–190]. XIAP experiences a copper-induced conformational change that promotes the ubiquitination of CCS at Lysine 241, a residue located near the CXC binding motif (in the third domain), which has been suggested to enhance CCS chaperone activity and thus SOD1 activation [183]. Multiple regions in XIAP are believed to bind Cu(I), including the cysteine rich BIR domains and RING finger [191]. COMMD1 is believed to interact with ATP7B in regulating intracellular copper levels [192,193]. XIAP also regulates COMMD1 via ubiquitination, leading to degradation of COMMD1 and increased cellular levels of copper [184]. While the protein binds Cu(II) in vitro in a 1:1 stoichiometry via 2 histidines and a methionine, its \(K_d\) value of \(3-5 \times 10^{-6}\) M [194] is several orders of magnitude weaker than other cellular copper proteins. In the absence of data to support Cu(II) binding in vivo, the true nature of how, and if, COMMD1 participates in copper

\(^1\) CopA of \(P.\) syringae is not an ATPase.
transport remains unknown [195]. Other potential mammalian copper regulatory proteins may be involved in the PKC and MAPK signal transduction pathways, as they are activated by increased levels of copper and copper-induced ROS [196,197].

4. Comparison of copper proteins

The broad classes of copper proteins, (i) those that utilize the metal as a cofactor and (ii) those that traffic the metal, can be distinguished by the differences in their cellular location and copper coordination environments.

4.1. Localization of copper proteins

In bacteria, very few copper proteins are found in the cytosol [134,198]. Oxidoreductases are either exported or located instead in the periplasm, while the electron transport proteins are usually membrane bound (Fig. 11). In eukaryotes, electron transport proteins are located within the mitochondria, oxidoreductases are found within organelles or exported from the cell, while copper chaperones comprise the bulk of the cytosolic copper-proteome. Some exceptions include Cu/Zn SOD, which is located in the cytoplasm of eukaryotes and the periplasm of gram negative bacteria, and Cu-dependent transcription factors and regulatory proteins, which are located in the cytosol and the nucleus.

4.2. Coordination environment: high-coordinate/high affinity vs. high-coordinate/low affinity and low-coordinate/high affinity

Different types of copper proteins can also be distinguished by their coordination environments, particularly by the type and number of ligands found in the inner coordination sphere. Enzymes and proteins that utilize copper as a cofactor bind the metal in high affinity, high coordinate environments (i.e. coordination numbers 4–5) that prevent loss of the metal during redox cycling. Copper trafficking proteins, on the other hand, promote metal lability either by having low affinity binding sites with moderate coordination numbers (usually ~4), or by having fewer ligands (i.e. coordination numbers 2–3) that bind with high affinity. Both of these strategies retain the metal but allow transfer under appropriate conditions. Furthermore, the metal cargo is usually conveyed as the more labile Cu(I) oxidation state. The binding sites are dominated by methionine, histidine, and cysteine residues, as predicted by the HSAB principle. Each one of these amino acids has unique copper binding properties, including oxidation state specificity, affinity, and pH dependence. These properties have been exploited by nature when evolving different copper proteins that perform different functions. As histidine is the only borderline ligand, it has the ability to effectively bind both Cu(I) and Cu(II), as opposed to the soft methionine and cysteine ligands that more effectively bind Cu(I). Cysteine exhibits the strongest affinity for copper as a result of the electrostatic character of the coordinate covalent bond that is lacking in histidine and methionine.

Table 8
Examples of transcription factors and regulatory proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac1</td>
<td>S. cerevisiae</td>
<td>↓ [Cu], promotes expression of Ctr1 and Ctr3</td>
<td>[177]</td>
</tr>
<tr>
<td>Ace1</td>
<td>S. cerevisiae</td>
<td>↑ [Cu], promotes expression of CUP1, CR55 and SOD1</td>
<td>[178,179]</td>
</tr>
<tr>
<td>CsoR</td>
<td>B. subtilis, M. tuberculosis</td>
<td>↑ [Cu], promotes expression of CopA and CopZ</td>
<td>[180]</td>
</tr>
<tr>
<td>CueR</td>
<td>E. coli</td>
<td>↑ [Cu], promotes expression of CopA</td>
<td>[182]</td>
</tr>
<tr>
<td>Regulatory proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIAP</td>
<td>H. sapiens</td>
<td>↑ [Cu], ubiquitinates CCS enhancing activity, ↑ [Cu], ubiquitinates COMMD1</td>
<td>[183]</td>
</tr>
<tr>
<td>COMMD1</td>
<td>H. sapiens</td>
<td>Ubiquitination and subsequent degradation results in ↑ [Cu]</td>
<td>[184]</td>
</tr>
</tbody>
</table>

Fig. 11. Location of copper proteins of interest in yeast and bacteria: a) is a diagram of a yeast cell and b) a generic bacterial cell. Most cytosolic copper proteins are trafficking proteins, with the exception of SOD1. Abbreviations: metallothionein (MT), outer membrane (OM), inner membrane space (IMS), inner membrane (IM), nitrite reductase (Nir), copper amine oxidase (CuAO), tyrosinase (Tyr), nitrous oxide reductase (N2OR).
interactions with copper. Histidine and cysteine bind copper via protonatable side chains with pK\textsubscript{a} values for the free amino acids of 6 and 8, respectively, and thus exhibit pH-dependent binding affinity.

As we have observed, the coordination chemistry of the electron transfer proteins and the oxidoreductases is dominated by higher coordination environments comprised of mostly histidine residues. Histidine can accommodate cycling between both oxidation states of copper without a considerable decrease in binding affinity that may result in the metal vacating the binding site. Cysteine is also commonly found in these binding sites, presumably to increase affinity for Cu(I), preventing metal loss. Methionine is rare in these binding sites and when found is usually a weakly coordinated axial ligand.

Copper ATPases and chaperones also bind Cu(I) with high affinity, but they do so in low coordination binding sites that enable copper transfer via ligand exchange reactions to a recipient protein. Copper ATPases and chaperones use 2 cysteine residues that bind copper in a high affinity ($K_D = 10^{-17}$–$10^{-19}$ M) [83,199], near linear geometry.

Copper resistance proteins are similar to the Cu ATPases and chaperones as they utilize binding sites that promote metal transfer, however, they do so with higher coordination numbers. What differentiates their higher coordination sites from those of the electron transfer proteins and oxidoreductases is ligand composition. Copper resistance proteins contain methionine-rich sites that are sometimes accompanied by a single histidine, as opposed to the multiple cysteine and histidine residues in electron transfer proteins and oxidoreductases. The resistance proteins exploit these methionine and histidine residues, which have a relatively weak Cu(I) binding affinity compared to cysteine, to promote metal lability ($K_D = 10^{-5}$–$10^{-12}$ M) [148,161,200]. The function of these resistance proteins is to move copper as cargo, in these cases cargo intended for export, not utilize it directly for function; hence the need to maintain labile metal binding sites.

The copper binding transcription factors have similar binding sites to the Cu ATPases and chaperones in terms of composition, however, metal lability here may be disadvantageous considering their function as cellular copper sensors. It is not known if these proteins acquire Cu(I) directly from other proteins or just scavenge “free” copper; likewise, it is unclear whether they transfer their cargo directly to other proteins after gene activation [201]. Unlike the copper transport, resistance, and chaperone proteins, the function of these regulatory proteins is not to traffic copper to other destinations, but rather sense changing levels of cellular copper and respond by activating other proteins to handle the changing copper status. These proteins employ various strategies for metal sensing and retention, including higher coordination number, less solvent accessible binding sites, and tighter binding affinity. As EXAFS data suggest, cysteine-rich Ace1 and Mac1 may reduce lability by increasing the coordination number, and possibly binding affinity, by coordinating Cu(I) with an additional cysteine residue [186]. Structural analysis of CsoR and CueR suggests that these proteins bind copper in less solvent accessible environments compared to other cytoplasmic copper transport proteins. CsoR and CueR also bind Cu(I) with tighter affinity than copper ATPases and chaperones ($K_D = 10^{-17}$–$10^{-19}$ M); CsoR $K_D < 10^{-19}$ M [181] and CueR has been described to have zeptomolar ($10^{-21}$ M) sensitivity [182]. Interestingly, CsoR also binds divalent metal ions with high affinity, but such non-native metal binding does not inhibit protein–DNA binding and therefore does not regulate gene expression [180]. This important observation demonstrates that the selectivity of sensing cellular copper over other metal ions is related not just to metal–protein binding affinity, but also to the intricacies of the metal–protein coordination environment that somehow couple metal–protein binding to protein–DNA binding. This allosteric regulation provides another level of control for handling a potentially toxic component like copper.

The high affinity copper transport proteins, or Ctr proteins, are perhaps some of the most diverse in terms of the variety of binding sites found in different versions expressed across the eukaryotic branch of life. While yeast and human Ctr1 (yCtr1 and hCtr1, respectively) have been the most studied, both structurally and biologically, a fair amount of biology is also known about the green algae version of Ctrl1 (crCtrl1). Each of these organisms binds copper with motifs of varying composition, yCtr1 with just methionine residues, hCtrl with histidine and methionine, crCtrl1 with cysteine and methionine; however, all three copies also contain a conserved methionine motif in the second transmembrane domain involved in transport of copper through the pore. The N-terminal, extracellular binding sites are most similar to those of the copper resistance proteins in that they bind copper with low affinity, presumably to facilitate movement of copper along the distribution network. They differ in mode of binding: the resistance proteins bind copper in highly organized binding sites with amino acids that can be of considerable distance from each other in primary sequence, while the Ctrl1 proteins bind copper in flexible contiguous motifs. The C-terminal binding sites are more similar to those observed in the intracellular copper proteins with coordination environments dominated by cysteine residues, with the occasional histidine. These are also presumed to be labile binding sites can hand off copper to the copper chaperone proteins.

4.3. Met vs His vs Cys: insights from Ctrl1 model peptides

The methionine and histidine motifs observed along the N terminus of the yeast and human versions of the Ctrl1 protein have been the topics of recent publications from our group [82,202–205]. Using model peptides, we have shown that Mets motifs of the type MX\textsubscript{2}MX\textsubscript{2}X\textsubscript{2}M are specific for Cu(I) and Ag(I) with no affinity for divalent metals, and they require at least 3 methionine residues for effective copper binding. Furthermore, their affinity for Cu(I) is independent of pH, motif arrangement, and composition of intervening amino acids. These relatively unstructured binding sites are considerably weaker when compared to the previously mentioned systems, with effective $K_D$ values ranging 2–11 $\times 10^{-6}$ M. This is biologically reasonable as the estimated $K_D$ for yCtrl-mediated transport is 1–5 $\times 10^{-6}$ M [71,75]. These studies have shown that methionine-rich sites provide a unique environment that stabilizes Cu(I) and provides exquisite selectivity over competing, biologically-relevant metal ions.

Mets motifs may not be sufficient for copper acquisition by all organisms under all conditions, however. Unlike yeast Ctrl1, which has exclusively methionine domains along its N-terminus, human Ctrl1 also has histidine clusters that are conserved throughout several mammalian species. We therefore explored the copper coordination chemistry of a model peptide containing the first 14 residues of human Ctrl1: MDHSHHMGMSYMD. We found that the two adjacent histidine residues along with an additional histidine or methionine bind Cu(I) much tighter than typical Mets motifs, with effective $K_D$ values at pH 7.4 between 6 $\times 10^{-11}$ and 2 $\times 10^{-13}$ [82]. This tighter binding must be balanced by the fact that metal coordination will be pH-dependent because of the histidine residues. Such a Cu(I) binding motif would therefore not be effective under acidic pH values. The presence of an ATCUN motif (see Fig. 8) means that this peptide can bind Cu(II), a function not accessible to the Mets-only motifs in yeast. Furthermore, the presence of the HH site facilitates the ascorbate-induced reduction of the peptide–Cu(II) complex to stabilize it in the Cu(I) oxidation state. Others have also previously recognized that HH sites impart unique redox properties to peptides [206–208]. Incorporation of histidine clusters among Mets motifs therefore modulates several chemical properties that are likely significant for biological copper acquisition, notably: pH-dependent binding affinity, adjustment of redox properties, and the ability to bind both Cu(I) and Cu(II).

We recently published a detailed analysis on how the individual chemical characteristics of the amino acids methionine, cysteine, and histidine affect Cu(I) binding by model Mets motif peptides of the sequence MG\textsubscript{x}MG\textsubscript{x}M, where $X$ is either Met, His, or Cys [204]. Comparison of the relative Cu(I) bindings affinities revealed that at
neutral pH Cys binds more tightly than His, and His more tightly than Met (pH 7.4 Cys > His > Met); at acidic pH Cys still exhibits the greatest affinity, however, Met binds more tightly than His (pH 4.5 Cys > Met > His). Cys is the most susceptible to oxidation, followed by Met, with His the most resistant to oxidation (Cys > Met > > His). Together, these findings highlight how the chemical properties of these amino acids, notably their susceptibility to oxidation and how their metal binding affinity depends on pH, provide preferences for use of certain amino acids over others in certain environments. For example, incorporation of cysteine is ideal for copper accumulation in hypoxic environments where the metal is scarce, as is the case for certain green algae [209]. Methionine, on the other hand, is well suited for copper accumulation in acidic, oxidative environments, as is the case for yeast [210] and likely in the human small intestine. Histidine would be preferred in neutral, oxidative environments where methionine-rich motifs may not bind tight enough, which may be the scenario in the extracellular environment of higher organisms.

5. Conclusions and implications

As we have seen in the previous discussion, the total number of ligands, their spatial arrangement, solvent accessibility and the various combinations of imidazole, thiolate, and thioether donor groups, all work together to provide binding sites for copper that either enable it to carry out a function, or safely transport it in a way that prevents toxic reactivity. While the focus of this review has centered on the inner-coordination spheres of copper, the effects are predicted to emanate beyond the local binding site to influence the larger picture of metal incorporation and utilization at the systemic level.

Questions of how proteins acquire the correct metal cofactor in vivo and how the metalloproteome is maintained as a system remain intense areas of research. Improper metal loading usually results in protein misfolding and loss of function. Metalloproteins in vitro coordinate metal ions with affinities largely reflective of the Irving-Williams series (Mn2+<Fe2+<Co2+<Ni2+<Cu2+<Zn2+), with Cu2+ being the most competitive [9]. Systemic processes must therefore promote protein acquisition of the correct cofactor in vivo while preventing copper from overwhelming the metalloproteome. In addition to the previously discussed mechanisms of the copper import and efflux pathways, copper chaperone networks, and metal sensing transcription factors, metal compartmentalization for protein maturation and maintenance of differential cellular levels of competing metals are emerging as critical components of these processes [211].

The properties of the various copper-binding motifs we explored in this review are likely to be important factors in how copper trafficking functions within and between different compartments. The pH dependence on copper binding revealed in the studies of Ctr1 model peptides described above provides one template of how various motifs could be optimal for binding or releasing copper depending on a local environment within a particular compartment [204]. Another study of a model peptide, this one containing the MXCXXC motif conserved in Cul(I) chaperone proteins, found by NMR that the Met residue participates in Cul(I) binding at acidic to neutral pH. This result contrasts the established binding mode between the two Cys residues, as described in Section 3.2, but provides the intriguing hypothesis that the pH sensitivity of Cul(I) binding might be functionally relevant [212]. Another thought-provoking discovery is that a luminal loop of the human copper-transporting ATPase ATP7A contains a Met/His-rich region that may be critical for transferring copper to acceptor proteins in the secretory pathway [213]. The loop contains the conserved sequence MDHxHx4-HxHxxxMSxexMx4-HxSlM, which is highly reminiscent of the N-terminal domain of human Ctr1 described above (MDHSHHMGHMMNDS) because of the inclusion of both Met and His motifs. The loop undergoes conformational transitions upon binding 2–3 copper, and stabilizes Cul(I) in what appears to be a highly dynamic environment conducive to copper transfer [213]. The results from our own work, combined with these studies from other labs, raise an interesting hypothesis that Met/His or Met/Cys combinations provide organisms with dynamic, multifunctional domains that can facilitate copper transfer under different extracellular, subcellular, and tissue-specific scenarios of pH, redox environment, and presence of other copper carriers or target proteins.

The elements of metalloregulation in complex multicellular organisms remain to be fully elucidated. Intriguing questions remain, for example, in how extracellular copper is maintained, regulated and partitioned systemically, and in how diseases affect (or are affected by) copper homeostasis mechanisms, just to name a few. The coordination chemistry principles elucidated here will likely be relevant to answering such questions.

Abbreviations

APP amyloid precursor protein
ATCUIN amino terminal copper/nickel binder
ATP adenosine triphosphate
CcO cytochrome c oxidase
CCS copper chaperone for SOD1
EPR electron paramagnetic resonance
EXAFS extended X-ray absorption fine structure
HSAB hard/soft acid base
IMS inner membrane space
LFSE ligand field stabilization energy
LFT ligand field theory
NMR nuclear magnetic resonance
RND resistance node division
ROS reactive oxygen species
SOD1 Cu, Zn superoxide dismutase (Cu, Zn SOD)
T1Cu Type I copper center
T2Cu Type II copper center
T3Cu Type III copper center

Acknowledgments

We thank the National Science Foundation (Grant 0449699), the Sloan Foundation and the Camille and Hanny Dreyfus Foundation for supporting our work in this area.

References
