Perspectives in Inorganic Structural Genomics: A Trafficking Route for Copper

Fabio Arnesano,[a] Lucia Banci,[a] Ivano Bertini,*[a] and Simone Ciofi-Baffoni[a]

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Metal ions are essential for living organisms because they are involved in many fundamental biological processes. Increasing evidence indicates that there are no free copper ions in the cell; however, a recently discovered category of proteins (metallochaperones) is responsible for copper ion trafficking and delivery to either specific target enzymes or to membrane ATPases for metal translocation. A search in the available genomes for copper chaperones and soluble domains of the ATPases reveals a large variety of systems. In this Microreview, the example of copper transfer from outside a yeast cell into the Golgi organelle is considered, as well as related processes in other eukaryotes and in prokaryotes. The investigation of the structure and metal-binding properties of these proteins is crucial for understanding the process of copper trafficking at the molecular level and how coordination chemistry may contribute to the interpretation of the energetics of the metal environment.

Introduction

Life developed in an inorganic matrix, i.e. the Earth's crust. Living organisms have since learned how to acquire the correct amounts of essential inorganic elements and how to defend themselves from unnecessary or poisonous ones. Metal ions need a number of proteins both in order to be acquired/excreted, and in order to be transported from the outside of the cell to the final target, which is a metalloprotein.[1] Such processes are performed by soluble proteins[2−4] and membrane proteins, such as pumps[5−7] and permeases.[8] Such proteins are encoded in genomes or plasmids, but it is not obvious from a genome analysis which proteins are involved in the above processes. What can be done, however, is to search in the gene bank proteins similar to those already known to bind metal ions.[9,10] The identification of a metal-binding motif (i.e. a pattern of conserved amino acids acting as ligands) can be exploited in the search.[11] Another tool for the identification of proteins

[a] Magnetic Resonance Center CERM and Department of Chemistry, University of Florence,
Via Luigi Sacconi 6, 50019, Sesto Fiorentino, Florence, Italy
Fax: (internat.) + 39-055-4574271
E-mail: bertini@cerm.unifi.it

Fabio Arnesano (far right) was born in Lecce, Italy in 1973. He studied Chemistry at the University of Florence where he received his laurea degree in 1997 and his Ph.D. in 2000. He is currently a postdoctoral fellow at the Magnetic Resonance Center of the University of Florence. His research is devoted to NMR spectroscopy and biophysical characterization of metalloproteins and protein complexes involved in electron transfer and metal-trafficking processes, with a focus on heme and copper proteins.

Lucia Banci (center left) is Professor of Chemistry at the University of Florence, Italy. Her research interests are devoted to structural characterization of biological molecules through NMR spectroscopy, the methodological aspects of its application to proteins containing paramagnetic metal ions, computational simulations of protein structure and dynamics, and genome browsing and functional genomics.

Ivano Bertini (center right) was born in 1940, obtained the Italian degree of Doctor in Chemistry at the University of Florence in 1964 and then the Libera Docenza in 1969. He became full professor in General and Inorganic Chemistry in 1975 at the University of Florence, where he is now. He received the Laurea Honoris Causa from the University of Stockholm in 1998, from the University of Ioannina in 2002 and from the University of Siena in 2003. He is a member of the Academia Europaea and of the Italian Accademia dei Lincei. Following his initial studies on theoretical/physical inorganic chemistry, he has, since 1975, been studying the structure-function relationships of metalloproteins. In 1994, he transformed his laboratory into an NMR laboratory for studying the structural biology of metalloproteins. He has pursued the advancements of technology for determination of solution structures (particularly of paramagnetic metalloproteins), and he has developed a molecular biology department for protein expression and purification.

Simone Ciofi-Baffoni (far left) was born in Florence, Italy in 1971. He obtained his Ph.D. degree from the University of Florence in 2000. He is now a postdoctoral fellow at the same university. His research interest is focused on the structural and dynamic characterization of metalloproteins through NMR spectroscopy. His current research is particularly devoted to copper trafficking proteins to elucidate molecular mechanisms of copper homeostasis.

MICROREVIEWS: This feature introduces the readers to the author's research through a concise overview of the selected topic. Reference to important work from others in the field is included.

involved in processing metal ions is that of looking at bacterial genomes — often proteins that interact with each other are close in these genomes or they are part of a segment of DNA — called an operon — which encodes a number of interacting proteins, plus their regulator(s) and promoter(s).[12,13]

An example of this is provided by the *cop* operon of *Enterococcus hirae*,[14] responsible for copper uptake, availability, and export in the bacterium by encoding a repressor (CopY), a copper chaperone (CopZ), and an import and an export copper ATPase (CopA and CopB, respectively).[14] Understanding of the mechanisms of metal transport and regulation at the molecular level is crucial in order to shed light on a number of biological processes, including the functional defects of genes. In so doing, a lot can be learned about this area of biological inorganic chemistry, which represents a window for the expansion of the field of inorganic chemistry. The most successfully studied system is that regarding copper — as a case study, this review will be devoted to one of its trafficking pathways.

Copper can adopt two distinct oxidation states, either oxidized (Cu^{II}) or reduced (Cu^{I}). Copper proteins are involved in vital processes such as respiration, iron transport, oxidative stress protection, blood clotting and pigmentation.[15] However, because of its redox activity, free copper would be highly toxic, even at low concentrations. Therefore, free intracellular copper is absent, and its concentrations need to be regulated within very narrow limits.[16] Disruption of copper homeostasis has recently been implicated in disease states and pathophysiological conditions.[17–19]

Results and Discussion

Copper in Yeast: From the Outside to the Multicopper Oxidase Fet3

Genetic experiments with baker's yeast *Saccharomyces cerevisiae* resulted in the cloning of the first gene encoding a cellular membrane protein associated with high-affinity Cu transport (Ctr), denoted Ctr1.[20] Indeed, yeast cells lacking a functional Ctr1 gene are unable to load Cu onto cytochrome *c* oxidase.[21] Copper uptake through Ctr1 is preceded by reduction of Cu^{II} to Cu^{I}, a process mediated by the cell surface metalloreductase Fre1[22] (Figure 1).

All members of the Ctr1 family of Cu transporters have three potential transmembrane domains, a hydrophilic amino terminus typically rich in methionine residues, and a number of other conserved residues of as yet unknown functional importance.[8] The amino-terminal region (located outside the cell) of baker's yeast Ctr1 harbors eight repeats of the putative Cu-binding motif MXMXXM (M = methionine, X = generic amino acid). The carboxy-terminal region (located inside the cell, in the cytosol) of Ctr1 is rich in charged amino acids and contains quite well conserved cysteines.[8] Thus far, studies reveal that both the yeast and human Ctr1 proteins are present as homo-multimeric proteins, with high affinity for Cu.[23,24]

Several proteins are, on the other hand, implicated in low-affinity trans-membrane copper uptake in baker's yeast, including Fet4 and Ctr2. Fet4 has no significant homology to the Ctr family of high affinity copper transporters. However, biochemical and genetic evidence suggests that Fet4 is able to transport both iron and copper.[25] Ctr2, which bears homology to Ctr1, may be localized in the membrane of organelles present inside the cell, and may play a role in the mobilization of copper ions[26] between different locations inside the cells.[27] O'Halloran et al. have demonstrated that there is no free copper in a cell,[16] and therefore copper, just like other metal ions, needs to be accompanied to different cell compartments or to its final destination by proteins called chaperones, responsible for copper distribution inside the cytoplasm.[2,3] The carboxy terminus of Ctr1 contains two CXC motifs which are proposed to bind Cu^{I} and exchange it with metallochaperones.[28] In support of this, it has been recently observed that a C-ter-

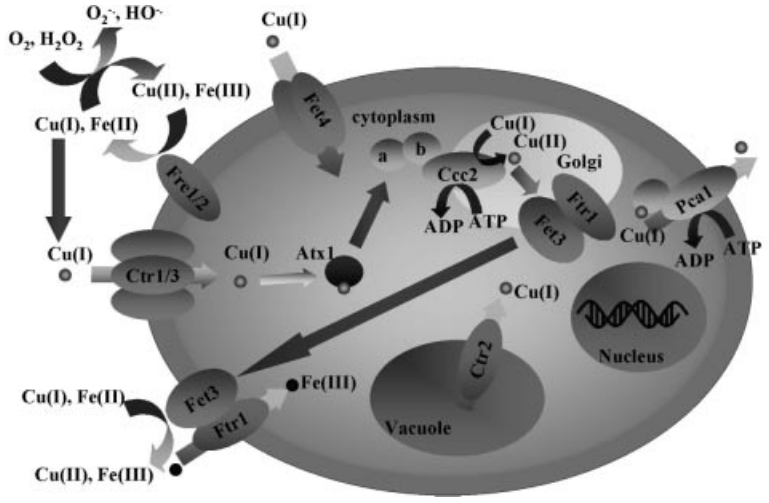

Figure 1. Some copper trafficking pathways in the yeast cell; depending on the extracellular copper concentration, copper can be transported inside the cell by two high-affinity copper transporters Ctr1 and Ctr3, or by a low-affinity Cu/Fe-transporter Fet4; Ctr2 could be involved in copper efflux from the vacuole; delivery of copper involves the metallochaperone Atx1 that shuttles copper to Ccc2, a P-type ATPase located in the trans-Golgi network, and is responsible for copper translocation; once copper is in the lumen of the secretory pathway, it is loaded onto Fet3, a multicopper-ferroxidase essential for high affinity iron uptake that partners with the Ftr1 protein

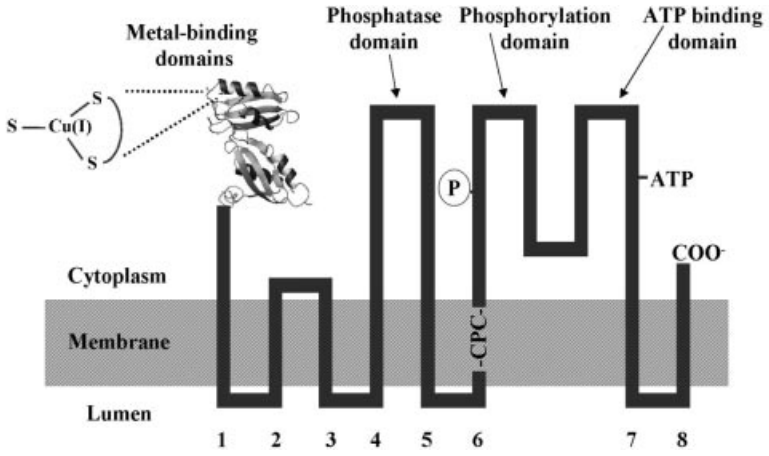

Figure 2. Schematic model of the copper-transporting ATPase Ccc2 from *S. cerevisiae*; the predicted transmembrane segments and the cytosolic domains are shown; "P" indicates a phosphate group; the copper binding motifs CXXC and CPC are also indicated; a similar structural organization is shared by all heavy metal P-type ATPases, but the number of N-terminal metal-binding domains is variable

minal domain of yeast Ctr1 exchanges Cu^{I} rapidly with the copper chaperone Atx1.[28] Atx1 delivers copper to the soluble copper domains of Ccc2, a P-type ATPase located in the Golgi membrane [29,30] (see Figure 1), which transfers copper to enzymes inside the Golgi organelle.[31] On the contrary, a different ATPase (Pca1), located on the cell membrane, appears to be involved in copper export from yeast.[32]

Ccc2 and Pca1 belong to the family of cation-transporting P-type ATPases (see Figure 2), which contain eight transmembrane segments, an ATP binding/phosphorylation domain (between transmembrane segments 6 and 7) and a phosphatase (or actuator) domain (between transmembrane segments 4 and 5).[5−7] Copper ATPases possess a variable number of N-terminal soluble metal-binding domains and a conserved CPx [C = cysteine, P = proline, x = C (cysteine) or H (histidine)] motif in transmembrane segment 6. Ccc2 has two soluble domains of about 70 amino acids, each containing a Cu^{I} binding CXXC motif. Pca1 contains only one domain preceded by a Cys-rich region of about 400 amino acids.[32]

Once Cu^{I} is bound to one or two soluble domains of Ccc2, it is transferred inside the Golgi organelle. It has been suggested that, during this transfer, Cu^{I} is oxidized to Cu^{II}, consistent with the more oxidizing environment of the Golgi organelle.[33] Copper is then loaded onto Fet3, a multicopper-ferroxidase essential for high-affinity iron uptake. When loaded with copper, Fet3 makes a complex with the iron transporter Ftr1[34] and, together, they move towards the cell membrane,[35] where Fet3 catalyzes the oxidation of Fe^{II} to Fe^{III}, which is then bound by Ftr1. Ftr1 and Fet3 are primarily expressed in oxygenated cultures, whereas anaerobic conditions induce the low affinity copper/iron transporter, Fet4.[36] Very recently, it has been observed that Fet3 also has a cuprous oxidase activity at the cell membrane, thus buffering the Cu^{I}/Cu^{II} ratio through an interplay with the cupric reductase Fre1.[37] By removing the Atx1 gene, iron uptake does not occur any more, as a consequence of failure to provide the metal cofactor to Fet3. These data, as well as other experimental evidence, point out at the tight interplay between copper and iron metabolisms. Figure 1 shows the mechanism by which Cu^{I} is acquired by Ctr1, taken by Atx1, released to the membrane protein Ccc2 and transferred to Fet3.

The structures of reduced apo- and Cu^{I}-loaded Atx1 were solved by NMR spectroscopy in Florence in collaboration with the Northwestern University[38] (Figure 3, top).

The solution structure of *S. cerevisiae* Cu^{I}-Atx1 exhibits a βαββαβ-fold with the Cu^{I} ion coordinated by two cysteine residues, Cys 15 (in loop 1) and Cys 18 (in helix α1), of a conserved CXXC motif. This fold is the same as that found by X-ray diffraction of the Hg^{II} derivative.[39] Atx1 shares significant sequence similarity (30 % residue identity) with a periplasmic mercury resistance protein, MerP.[40] Both the MerP solution structure[41] and the Hg^{II}-Atx1 crystal structure[39] show an Hg^{II} ion coordinated by the sulfur atoms of the two cysteines of the CXXC motif with a linear coordination geometry. In contrast, the solution structure of Cu^{I}-Atx1 has an average S−Cu−S angle of 120±40°, suggesting that Cu^{I} prefers a coordination number higher than two. EXAFS analyses of Cu^{I}-Atx1[42] and of its homologous protein, CopZ, from *Bacillus subtilis*[43] and *Enterococcus hirae*[44] showed that a third sulfur atom completes the coordination sphere of Cu^{I}, indicating its preference for a three-coordinate environment. A conserved Met residue, two residues upstream of the CxxC consensus motif, is a candidate for this third ligand. However, in the structures of Cu^{I}-Atx1 and Cu^{I}-CopZ from *B. subtilis*, the average positions of the Met sulfur atoms are 7.0±1 Å and 6.2±1 Å from copper, respectively, which rule their involvement in copper coordination.[38,45] Even in the Hg^{II}-Atx1 crystal structure[39] the Met sulfur is too far from copper (8.1 Å), suggesting it is not involved in metal coordination. The exposed location of the metal site suggests that the third ligand may be provided by an external thiol group.

These proteins are prepared by incubation of the apoprotein with a Cu^{I} complex in the presence of a thiol reductant, dithiothreitol (DTT).[29] In vivo, DTT is likely to be

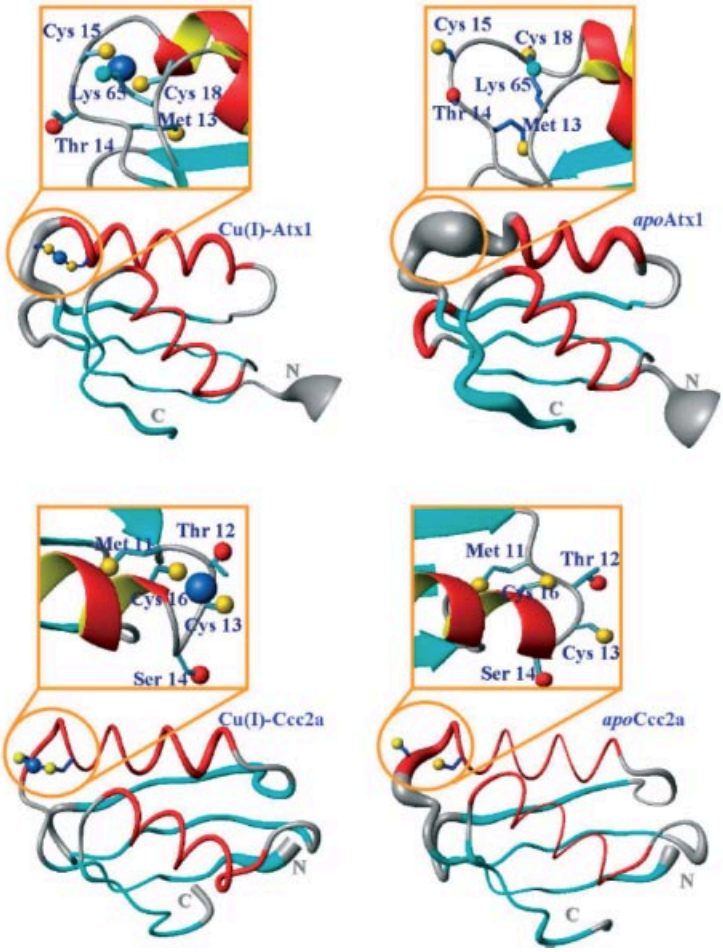

Figure 3. Solution structures of Cu^I- and apo-loaded Atx1 (top left and top right, respectively) and Cu^I- and apo-loaded Ccc2a (bottom left and bottom right, respectively); the structures are represented as a tube whose radius is proportional to the structural resolution; helices are colored in red and β-strands are colored in cyan; the insets show the copper binding site where copper is represented as a blue sphere; copper-binding cysteines and other neighbouring residues are also indicated

replaced by glutathione, which is present at high concentration (about 5 mM) inside the cell. When copper reduction is carried out with ascorbate, the EXAFS spectra of Cu^I-CopZ indicate that one oxygen atom of ascorbate is present in the first coordination sphere of copper, together with the two sulfur atoms of the Cys residues.[43] The three-coordinate environment of copper, with one ligand being exogenous and easily detectable, has a crucial role in the metal transfer process (see later).

The overall folding of apoAtx1 is essentially identical to that observed for the copper-bound form of the protein, with the exception of helix α1, which is shorter by one helical turn at the N-terminus.[38] Comparison of the Cu^I- and apo-conformations of the protein also reveals that the Cu^I-binding cysteines move from a buried site in the metal-bound form to a solvent-exposed conformation on the surface of the protein, after copper release (see Figure 3, top). Slight structural changes, involving both the charged and hydrophobic residues close to the metal-binding site,[38] may signal to the partner whether the metallochaperone is in the demetalated or in the copper-loaded form, and may trigger binding of Atx1 to Ccc2.

The structure of the first domain of Ccc2 (Ccc2a) was solved by NMR spectroscopy, and exhibits a βαββαβ, ferredoxin-like fold similar to that of its partner Atx1[46] (Figure 3, bottom). The overall fold of Ccc2a remains essentially unchanged upon copper loading. Unlike Atx1, only few conformational changes are observed in Ccc2a upon copper release, and the metal-binding region (including the conformation of the metal-binding cysteines), is well defined in both apo and holo forms, suggesting that the metal site in apoCcc2a is pre-organized to some extent to receive the copper ion.[46] Again, the structure indicates the copper environment is three-coordinate (S−Cu−S average angle 120±30°).

Modeling of the whole N-terminal region of Ccc2, encompassing the two soluble domains, was performed on the available structure of the homologous protein from *B. subtilis*,[47] and suggests that the two copper binding sites are far apart and cannot interact with each other. However,

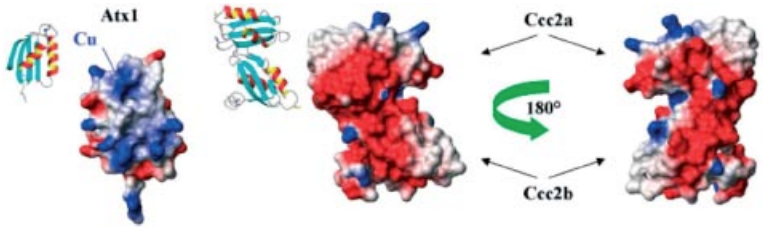

Figure 4. Electrostatic surface potential of Cu^{I}-Atx1 from *S. cerevisiae* and of a structural model of the two soluble domains of Ccc2 from *S. cerevisiae* based on the structure of CopAab from *B. subtilis*; the ribbon representations show the orientation of the two proteins; the copper binding cysteines of the CXXC motifs of Atx1 and Ccc2 domains are indicated in yellow; copper is shown as a blue sphere

they can interact with Atx1. Indeed, the surface of Atx1 is positively charged, whereas those of the Ccc2 domains are negative (Figure 4).[48] The electrostatic interaction is the long-distance driving force which brings the two partner proteins together.

The protein surface interacting with the partner can be monitored by NMR spectroscopy, through the mapping of $^{1}H/^{15}N$ chemical shift changes occurring in both proteins when titrated with the partner [Figure 5(A)].[48,49] These residues are located in loops 1 and 5, helix α1, and the C-terminal part of helix α2 in both proteins. Besides the metal-binding cysteines and the charged amino acids, some polar and hydrophobic residues are involved in interactions at the interface. As mentioned above, the hydrophobic patches at the protein interface are different between the apo and the copper-loaded forms of Atx1, whereas Ccc2a maintains a similar conformation and pattern of hydrophobic contacts around the metal-binding site, regardless the metalation state. Comparison with the structures of homologous proteins confirms that in most of the cases metal binding affects a hydrophobic patch around the metal site in metallochaperones, possibly for tuning and optimizing the hydrophobic interactions with the ATPase domains.[50]

Thermodynamic and kinetic considerations suggest that the copper chaperones are designed to increase significantly the rate of copper transfer between physiological partners and then to overcome the extraordinary copper chelation capacity of the eukaryotic cytoplasm. In this sense, metallochaperones work like enzymes, lowering the energetic barriers along a specific reaction pathway. The thermodynamic gradient for metal transfer between Atx1 and Ccc2 is shallow (K_{eq} = 1.5), establishing that transfer of copper from Atx1 to Ccc2 is not based on a higher copper affinity of the target domain.[51] Instead, Atx1 allows rapid metal transfer to the ATPase ($k_{ex} > 10^{3}\ s^{-1}$),[48] which, in turn, transfers copper across the membrane, thus driving the thermodynamic overall equilibrium. A low activation barrier for transfer between partners results from complementary electrostatic forces that orient the metal-binding loops of Atx1 and Ccc2 for formation of copper-bridged intermediates.[30,51]

As mentioned above, the exposed location of the metal-binding site allows copper to expand its coordination sphere, thus becoming three-coordinate. During the copper transfer process, the third labile copper ligand in Cu^{I}-Atx1 would be displaced by the first Cys of the CXXC motif on the partner molecule, to form a three-coordinate intermedi-

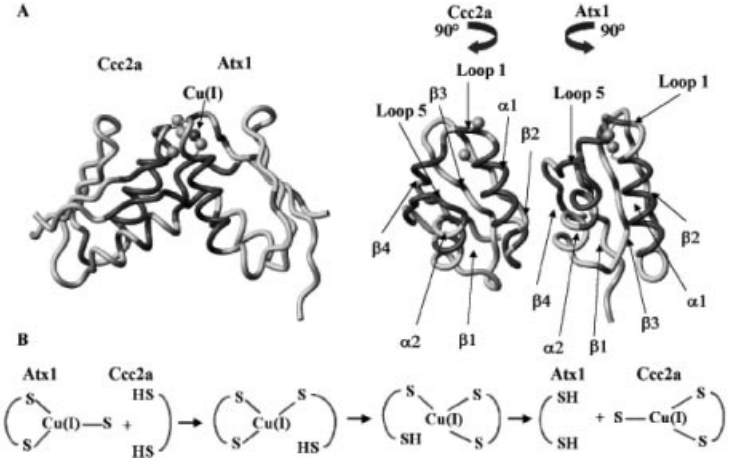

Figure 5. (A) The Ccc2a:Atx1 adduct model calculated using NMR chemical shift mapping; the view on the right-hand side shows the molecules rotated by 90° to expose the interaction surfaces; residues involved in the interaction are in dark grey; residues not involved in the interaction or not observed are in light grey; the copper ion is in dark grey and the sulfur atoms of the copper-binding cysteines are in light grey; secondary structure elements are indicated; (B) the proposed mechanism for copper transfer between Atx1 and a domain of the Ccc2 protein

ate. When the second Cys of Ccc2 binds copper, one Cys of Atx1 is detached, thus weakening the interaction of copper with Atx1. In the final step, the second Cys of Atx1 is detached, (probably) replaced by an exogenous ligand, and the metallochaperone leaves in its apo form (see B in Figure 5). These structural changes are accompanied by an increase in mobility of residues at the interface, which may favor complex dissociation after copper release.[48]

What Determines Metal Selectivity?

The βαββαβ structure containing a CXXC metal-binding motif has been adopted in nature to carry a variety of metal ions (Cu, Hg, Zn, Cd, Pb) with selective affinity.[52−54] Atx1 shares significant structure similarity with a periplasmic mercury resistance protein (MerP), which interacts with a membrane protein of the detoxification operon.[40] The solution structure of MerP[41] shows an Hg^{II} ion coordinated by the two cysteines of the CXXC motif, with a linear coordination geometry. It has also been shown that MerP is capable of binding to other divalent metal ions with lower affinities.[54,55] Similarly, the copper chaperone CopZ from *E. hirae* forms specific metal complexes with copper, mercury, cadmium and cobalt with the following order of affinity: $Cu^{I} > Hg^{II} > Cd^{II} >> Co^{II}$.[56] In addition, soluble domains of bacterial zinc-transporting[57] and cadmium-transporting ATPases,[58] either experimentally found[59] or predicted to have the βαββαβ fold, possess the CXXC binding motif. Therefore, it can be inferred that the metal-binding selectivity does not reside in the overall fold but in sequence variations close to the metal site.

For zinc ATPases, sequence analysis leads to the earlier suggestion that an Asp residue, which is found just before the N-terminal cysteine of the CXXC consensus motif, may modulate metal-binding affinity.[9] EXAFS data on the N-terminal domain of the zinc ATPase ZntA from *E. coli* indicate that Zn is coordinated by two sulfur atoms and two N/O atoms.[59] The structure of ZntA has been recently solved[59] and suggests that the conserved Asp residue can indeed coordinate zinc. The presence of three negative charges (one aspartate and two cysteines) ought to provide a more favourable electrostatic contribution for the binding of a Zn^{II} ion with respect to a Cu^{I} ion. Moreover, in terms of hard/soft acid-base theory, the Asp oxygen will stabilize binding of the harder (or less polarizable) Zn^{II} ion more than the highly polarizable Cu^{I} ion. In copper chaperone sequences, the position immediately preceding the N-terminal Cys in the metal-binding loop is often occupied by a Thr residue, which provides a second-shell interaction for copper.[60−62] These data suggest that the amino acid at this position plays a role in metal ion selectivity.

Other factors could determine metal selectivity of the CXXC metal-binding motif in a βαββαβ structure, such as the size of the binding pocket (which can be controlled by the tightness of the turn of the binding loop), or by electrostatics. The nature of the residues in the turn of the metal-binding loop affects their interactions with the solvent and with other protein residues, and can contribute to the turn tightness and/or to electrostatic properties.

Comparison of Homologous Proteins

The genome-sequencing projects developed in the last few years have made available the primary sequence of an enormous number of new genes. The draft sequence of the human genome[63] is now available, together with the complete genomes of many other organisms (see http://www.ncbi.nlm.nih.gov). Functional associations of neighboring genes can be detected by bioinformatic tools and can be used to identify potential protein partners.[12,13] This huge amount of incoming information has stimulated the development of Structural Genomics projects (http://www.spineurope.org/, http://www.nigms.nih.gov/psi/ and http://www.rsgi.riken.go.jp/), with the aim of elucidating the function of proteins through the determination of their three-dimensional structure. The idea can be translated in the motto: "*From sequence to function through structure*". Moreover, genome sequencing allows us to compare homologous sequences in different species, in an "orthologue" approach, and to model them when sequence identity with at least one protein of known structure is higher than 30 %.[64] Structure similarities allow the function of one family of proteins to be predicted from that of the other. In the case of metalloproteins, conserved residues forming the consensus motifs for metal binding can be used as selection criteria in a genome-wide search, together with sequence identity thresholds.[11] An orthologue search shows that proteins homologous to yeast Atx1 and Ccc2 are found in all the eukaryotes and in many bacteria.[9] Structural models can be calculated using the high-resolution solution structures of yeast proteins as templates, and structural modelling allows us to identify common structural features, such as the hydrophobic core, electrostatic potential surfaces and metal-binding site residues.

Searches have shown that bacteria are very diverse, and may develop alternative routes for copper homeostasis.[9] *B. subtilis* has a CopZ which acts as chaperone[45] as well as a CopA which is an ATPase containing two soluble metal-binding domains.[60] In this organism it has been shown that the CopZ-CopA interaction mechanism is used for copper excretion.[65] It is peculiar that CopZ has opposite overall charge with respect to its eukaryotic homolog Atx1 and, of course, opposite charges are displayed by the two metal-binding domains of the ATPase.[66] In *B. subtilis*, the first domain of CopA is not well folded in vitro, but a point mutation at position 46 is sufficient to fold the protein properly.[67] It is possible that the first domain is not folded in vivo or not used at all. Copper ATPases of other bacteria have indeed only one soluble domain. In *E. hirae* the homologous proteins are thought to function in copper uptake.[68] The *cop* operon of *E. hirae*, in addition to the CopA ATPase and the copper chaperone CopZ, consists of two more genes, one encoding a repressor (CopY), which, upon receiving copper from CopZ, breaks its interaction with DNA.[44,69] The other is a second P-type copper ATPase

(CopB), which is involved in the extrusion of copper when it reaches toxic levels[68] (Figure 6, top).

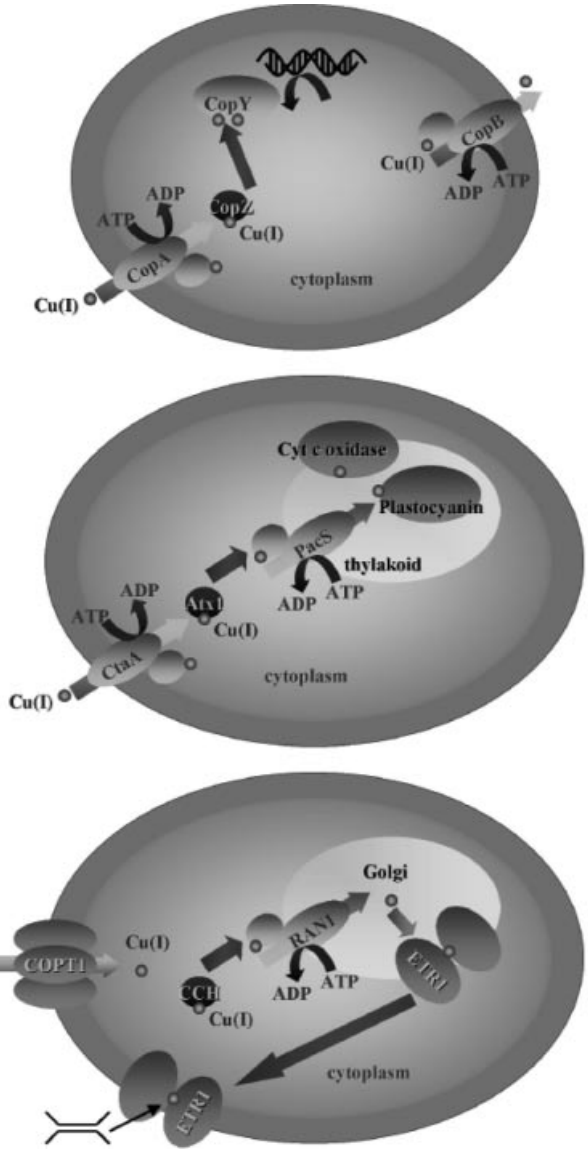

Figure 6. Copper-trafficking pathways in the bacterium *Enterococcus hirae* (top), in the cyanobacterium *Synechocystis* PCC 6803 (center) and in the plant *Arabidopsis thaliana* (bottom)

Some cyanobacteria have a peculiar cellular organization which involves organelles called thylakoids. These contain cytochrome *c* oxidase and plastocyanin, and both proteins require copper to function. In these bacteria, two copper-transporting ATPases are present, namely CtaA and PacS.[70] The former is located on the external cellular membrane and is involved in copper uptake, whereas the latter is on the thylakoid membrane and transports copper inside this organelle for its delivery to copper-requiring proteins.[71] Copper is shuttled between these two ATPases by a soluble small protein, which is able to interact with the soluble copper-binding domains of both ATPases.[70] Interestingly, this copper chaperone possesses a His residue on loop 5 in the position corresponding to Lys 65 in Atx1 from *S. cerevisiae*. The imidazole ring of the latter His is predicted to protrude from loop 5 of the cyanobacterial protein, providing a putative third copper ligand. This residue is proposed to influence the direction of metal transfer in the interaction of the chaperone with the amino-terminal domains of the two ATPases PacS and CtaA [72] (Figure 6, center).

Plants also need copper for a variety of functions. One of these functions is unique to this kingdom of life and involves binding of ethylene, an important endogenous plant hormone affecting many aspects of growth and development. Ethylene binding occurs through five copper-dependent ethylene receptors (ETR1, ERS1, ETR2, EIN4 and ERS2).[73] In *Arabidopsis thaliana*, one of the two plants for which the complete genome is available, a copper pathway similar to that of *S. cerevisiae* has been identified.[74] Using the yeast system as a guide, it has been postulated that copper enters the cell through COPT1, the homologue of yeast CTR2. Copper is shuttled by a soluble protein (CCH), the homologue of Atx1, to a copper ATPase (RAN1), which resides in the Golgi, like Ccc2.[74] Here, the ATPase delivers one copper ion to the ethylene receptors (ETR1 family), which thus become functional and move to the external cell membrane to sense ethylene concentrations.[74] Ethylene interacts with Cu^{I} in an electron-rich hydrophobic pocket formed by membrane-spanning helices of the ETR1 dimer[75] (Figure 6, bottom).

In humans, two P-type ATPases, Menkes (MNK)[76−78] and Wilson (WND)[79,80] proteins, and a copper chaperone (Hah1[81]), homologous to yeast Ccc2 and Atx1 respectively, are present. The key role of MNK and WND in the cellular copper trafficking is strongly determined by the intracellular localization mechanism by which these proteins manage to deliver copper to the recently synthesized copper proteins.[33,82] At low copper concentrations, both proteins are localized in the Golgi, where MNK transfers copper to lysyl oxidase, which is responsible for cross-linking collagen and elastin. At high copper concentrations, MNK moves to the external cell membrane and pumps out of the cell the excess of copper. When the intracellular copper levels are reduced, the protein returns to the Golgi membrane.[33] WND, also located in the Golgi membrane, incorporates copper (at low concentrations) into ceruloplasmin, a multicopper oxidase similar to Fet3. At high copper concentrations, WND moves from the Golgi membrane to a vesicular compartment where it releases copper. The copper-loaded vesicles are then discharged into the bile.[82]

Both proteins have six soluble metal-binding domains at the N-terminal region, in addition to an intramembrane potential metal-binding site containing a CPC motif. The soluble metal-binding domains were suggested to be involved in the copper-dependent regulation of WND. Indeed, the copper-bound N-terminal WND domains (1−6) interact with the ATP-binding domain less tightly than their apo forms, thus increasing the affinity of the domain for ATP.[83]

However, only binding of copper to the intramembrane CPC binding site is essential for the copper-dependent cata-

lytic activity of the ATPase. This dependence is supported by the fact that mutations in the CPC motif destroy the activity,[84] whereas mutations in any or all of the six soluble domains do not.[85] It has been recently shown that affinity of the intramembrane copper-binding site is regulated by binding of copper to the 5th and 6th domains of WND, while the first four domains of WND are not involved in this process.[86] The need for six domains is not certain. Bacterial and yeast homologues have only one or two domains, suggesting that most of the metal-binding domains are not essential for the copper transport function.[9] A unique feature of mammalian copper-ATPases is their ability to undergo copper-dependent vesicular trafficking. Therefore, metal-binding domains 1−4 can be involved in the copper-dependent regulation of the ATPase subcellular localization, i.e. when copper concentration is increasing and all six metal-binding sites become occupied with the metal, the conformation of the N-terminal region changes,[87] thus inducing the translocation of the protein from the Golgi to the external cell membrane[88] or to a vesicular compartment.[89] Here, the copper ATPases act like their bacterial homologues, pumping excess copper out of the cytoplasm, thus detoxifying the cell.

The six metal-binding domains of MNK and WND are connected by linkers of different length. The longest flexible linker separates metal-binding domains 1−4 from domains 5 and 6.[9,90] Interestingly, it has been observed experimentally that the copper chaperone Hah1 interacts preferentially with domains 1−4 of WND.[53] It has been therefore proposed that domains 5 and 6 may receive copper from domains 1−4.[90] When Cu^{I}-Hah1 is added in large excess, copper is transferred to all six domains of WND, but Hah1 can also function as a regulator, by removing copper from N-terminal domains.[91] Although a slight excess of apo-Hah1 is sufficient to remove the first four copper atoms, removal of the other two copper atoms cannot be completed even using a large excess of apoHah1. It has been suggested that copper that is strongly bound to N-terminal domains of WND is essential for enzymatic activity, whereas exchangeable copper is bound to regulatory sites.[91]

The structures of a number of single metal-binding domains of MNK are available.[61,92] The other domains of MNK and WND have been modeled.[9] All the domains share a βαββαβ fold but display different surface charges. Interestingly, MNK possesses a seventh predicted βαββαβ domain between the first and the second metal-binding domains, but lacks a CXXC motif. In WND, only a short linker separates domains 1 and 2.[9] The arrangement of the last two soluble domains of MNK and WND, (i.e. those closest to the membrane) is thought to be similar to that of CopA from *B. subtilis* and Ccc2 (see above).[47]

Mutations and Illnesses

The advances in knowledge of the function of MNK and WND have been catalyzed by the fact that they are involved in two human disorders of copper transport.[93] Mutations of MNK in humans cause a copper deficiency disorder (Menkes' disease), and mutations of WND result in a Cu toxicity condition (Wilson's disease). The reason for different diseases caused by mutations in proteins of similar structure is related to the involvement of the two proteins in different patterns of cellular trafficking and their distinct pattern of tissue expression.[88,89] The Menkes gene is expressed in most tissues; the Wilson gene is expressed mainly in the liver, where it is responsible for biliary excretion of copper.

Different types of mutations have been identified which alter the copper metabolism in the Golgi (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). These mutations are mainly located in the transmembrane portion of the ATPases, with a few present in the soluble metal-binding and ATP-binding domains. One of the most common disease mutations, a H1069Q substitution, causes intracellular mislocalization of WND.[94] His 1069 is located in the ATP-binding domain of WND and is conserved in all copper-transporting ATPases from bacteria to mammals; experiments indicate that His 1069 is responsible for proper orientation of ATP in the catalytic site of WND prior to ATP hydrolysis.[95]

Several mutations found in Wilson's disease patients were shown to disrupt the ability of WND to interact with the soluble copper chaperone Hah1, which is homologous to yeast Atx1. Three disease-associated mutations (G85V, L492S, and G591D mutations) in the soluble metal-binding domains of WND, determine a marked reduction in interaction with Hah1, suggesting that the inability of Hah1 to deliver copper to WND constitutes the molecular basis of Wilson's disease in those patients harbouring these mutations.[96]

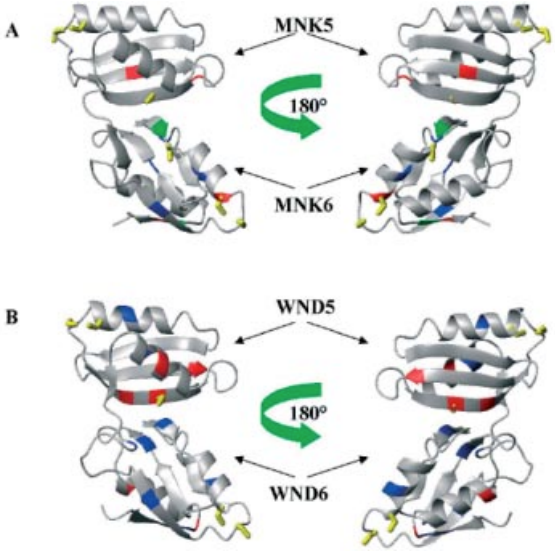

Figure 7. Structural models of the two linked soluble domains closest to the membrane (5 and 6) of the human Menkes (MNK) (A) and Wilson (WND) (B) proteins based on the structure of CopAab from *B. subtilis*; cysteine residues are indicated in yellow; mutations are coloured as follows: red = nucleotide substitutions (missense/nonsense), blue = small deletions, green = small insertions

The two domains closer to the membrane (5 and 6), have been shown to be essential for the functional activity of both MNK[97] and WND.[86,98] A structural model of these two domains can be obtained using the structure of CopAab from *B. subtilis*[47] as template. Significantly, although the length of the short linker between these two domains is not exactly the same for MNK/WND and CopAab,[9] residues at the interface between the two CopA domains are conserved in the last two soluble domains of both MNK and WND,[47] thus supporting a similar quaternary arrangement for this pair of domains. Mapping disease mutations on the models of these domains shows that some of them are farther from the two metal-binding sites, but they can affect the interaction between these two domains (Figure 7).

Concluding Remarks and Perspectives

The biochemistry of metal ions is becoming increasingly important, because of its tremendous implications in medicine and biology, as witnessed by the large number of proteins, emerging from genome sequencing and genetic studies, that are involved in diseases linked to metal ion homeostasis. Several copper-trafficking pathways involving metallochaperone-like proteins and P-type ATPases modulate copper ion homeostasis and delivery in bacteria and eukaryotes. These systems are at the boundary between inorganic chemistry and genomics, which has raised our interest as chemists working in the field of *inorganic structural biology*. Taking advantage of the large amount of structural information on yeast Atx1 chaperone and Ccc2 transporters and their interactions with metals, the field is moving towards a deeper understanding of homologous systems, with particular interest in the Menkes and Wilson ATPases involved in neurodegenerative disorders in humans. In these systems, it will be very useful to study the influence of the metal sites on the three-dimensional structures of proteins and multiple domain organization, in order to envisage the possible causes of degeneration and diseases. Finally, the mechanism of copper translocation across membranes needs to be fully elucidated.

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