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Functional Characterization of the Cellular Copper Proteome

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I hereby declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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Rakulise vase proteoomi funktsionaalne iseloomustamine

KAIRIT ZOVO

TTÜ KIRJASTUS

CONTENTS

INTRODUCTION

Copper has had and still has an enormous influence on the progress of human civilization as a metal. However, copper ions have played an even more important role in the evolution of life on Earth as a biometal. Ionic copper is used by almost all forms of current life. However, at the early stages of biological evolution when the Earth's environment was reducing, copper existed primarily in the form of insoluble Cu(I) sulfides and was not available for living organisms. Therefore, copper is a relatively new biometal, which became bioavailable only after the evolvement of oxygen into the Earth's atmosphere, which oxidized Cu(I) to Cu(II) and made copper soluble (Saito et al., 2003, Ridge et al., 2008). The redox properties of copper suited it ideally to exploit the oxidizing power of dioxygen for biological purposes (Crichton and Pierre, 2001). Copper ions have become essential components of cellular organisms, mediating important oxidative processes in the cell (Uauy et al., 1998).

Although the principal components of the cellular copper proteome are known, the principles of copper homeostasis have remained elusive. At the same time increasing evidence shows that dyshomeostasis of copper is closely linked to several neurodegenerative diseases as well as to normal aging (Gaggelli et al., 2006, Donnelly et al., 2007). Knowing the principles of cellular copper homeostasis might therefore help to combat these diseases as well as support healthy aging, which is extremely important considering the aging population.

The goal of this study was to gain a deeper understanding of the functioning of the copper proteome in the cell. In the literature overview the cellular copper proteome, consisting of copper-binding enzymes, copper transporters, and chaperones involved in copper trafficking within the cell are reviewed. After that the methods used for determination of Cu(I)-binding constants are reviewed and dissociation constant values for these Cu(I)-protein complexes have been collected from the literature. In order to characterize the functional proteome, the corresponding values should be systematically re-estimated under strictly controlled and similar conditions. In the Results and Discussion part the affinities of cellular copper-binding proteins determined by way of an ESI-MS-based approach are presented and discussed. The obtained results provide a more detailed insight into cellular copper trafficking and distribution.

ABBREVIATIONS

Atox1 – copper chaperone for P_{1B}-type ATPases, human homologue (HAH1)
ATP7A – Cu(I) P_{1B}-type ATPase defective in Menkes' disease
ATP7B – Cu(I) P_{1B}-type ATPase defective in Wilson's disease
BCA – bicinchoninic acid
BCS – bathocuproine disulfonate
Ccc2 – Cu(I) P_{1B}-type ATPase, yeast homologue
CCO – cytochrome c oxidase
CCS – copper chaperone for SOD
CopZ –copper chaperone for P_{1B}-type ATPases, prokaryotic homologue
Cox – copper chaperone for cytochrome c oxidase (Cox17, Cox19, Cox23, Cox11)
Ctr – high-affinity plasma membrane Cu(I) transporter
DETC – diethyldithiocarbamate
DTT – dithiothreitol
ESI-MS – electrospray ionization mass spectrometry
GSH – reduced glutathione
GSSG – oxidized glutathione
HAH1 – copper chaperone for P_{1B}-type ATPases, human homologue (Atox1)
HSQC – heteronuclear single quantum correlation
IMS – intermembrane space
ITC – isothermal titration calorimetry
MBD – metal-binding domain
MD – Menkes' disease
MNK – ATP7A or protein defective in Menkes' disease
MNK D1 – ATP7A or Menkes' disease protein N-terminal domain 1
MT – metallothionein
Sco1 and Sco2 – copper chaperones for cytochrome c oxidase
SOD – superoxide dismutase
SOD1 – copper,zinc-superoxide dismutase
SOD2 – manganese-superoxide dismutase
SOD3 – extracellular copper,zinc-superoxide dismutase
TCEP – tris(2-carboxyethyl)phosphine
WD – Wilson's disease
WND – Wilson's disease protein or ATP7B

ORIGINAL PUBLICATIONS

I Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., **Zovo, K**., and Palumaa, P. „Affinity gradients drive copper to cellular destinations“ (2010) Nature.;465(7298):645-8.

II Chung, R. S., Howells, C., Eaton, E. D., Shabala, L., **Zovo, K.**, Palumaa, P., Sillard, R., Woodhouse, A., Bennett, W. R., Ray, S., Vickers, J. C., West, A. K. "The native copper- and zinc-binding protein metallothionein blocks copper-mediated Abeta aggregation and toxicity in rat cortical neurons" (2010) PLoS One 5(8): e12030.

III Zovo K., Palumaa P. “Modulation of redox switches of copper chaperone Cox17 by Zn(II) ions determined by new ESI MS-based approach” (2009) Antioxid. Redox Signal.;11(5):985-95

1. REVIEW OF THE LITERATURE

1.1. Copper in the cell

In the cell copper ions are primarily involved in the utilization of molecular oxygen in the electron transfer chain and the generation of a biological energy reserve in the form of a proton gradient, which is further used for the production of ATP. Copper-containing cytochrome c oxidase (CCO) is the key enzyme in this energy supply system, already appearing in prokaryotic organisms (Musser and Chan, 1998). The most important step in cellular evolution was the symbiosis of a prokaryotic progenitor of mitochondria with a prokaryotic cell, leading to the development of mitochondria and the origin of the nuclear genome of the eukaryotic cell (Gray et al., 1999). Within this consortium the mitochondria have specialized in the utilization of oxygen and production of biological energy with the assistance of CCO.

However, given its redox reactivity, copper ions can also be harmful for living organisms by generating reactive oxygen species that may cause serious damage to all cellular components (Valko et al., 2005). Moreover, copper ions also have a high affinity for biological ligands and can replace other physiologically relevant metal ions – for instance Zn(II) – in the metal-binding sites of metalloproteins (Tottey et al., 2005). This makes an excess of copper, and especially nonspecifically bound copper ions, highly toxic to most living organisms (Andreini et al., 2008) and they require specific tools to combat the toxic side-effects of copper ions.

To combat superoxide radicals, a by-product of aerobic energy production, cells have developed a special enzyme called superoxide dismutase (SOD), which converts superoxide radicals to molecular oxygen and hydrogen peroxide. Two major kinds of SODs have evolved in prokaryotes: iron,manganese and copper,zinc SODs (Bordo et al., 1994, Zelko et al., 2002). All eukaryotic cells contain Cu,Zn-SOD and Mn-SOD, designated SOD1 and SOD2, respectively (Desideri and Falconi, 2003, Culotta et al., 2006). SOD1 resides in the cytoplasm as well as in the intermembrane space (IMS) of mitochondria, whereas SOD2 is present in the mitochondrial matrix (Furukawa and O'Halloran, 2006, Crichton and Pierre, 2001). In parallel with the exploitation of copper in mitochondrial CCO and SOD1, in eukaryotic cells complex systems for copper influx and the delivery of copper to target proteins were also created, which apparently also support the purpose of maintaining intracellular free copper under strict control.

Depending on extracellular copper concentrations, copper ions enter the eukaryotic cell through high-affinity plasma membrane copper transporters of the Ctr family (Ctr1 and Ctr3) (Dancis et al., 1994, Knight et al., 1996, Pena et al., 2000, Nose et al., 2006) and the low-affinity permeases Fet4 (Hassett et al., 2000) and Smf1 (Cohen et al., 2000). Human Ctr1-mediated copper transport is stimulated by extracellular acidic pH and high K^+ concentrations (Lee et al., 2002). Copper is delivered to the cell in the reduced Cu(I) form, and extracellular Cu(II) ions are reduced prior to cellular entry (Hassett and Kosman, 1995, Georgatsou et al., 1997). In prokaryotes copper enters the cell more easily (Lutkenhaus, 1977, Outten et al., 2001, Rensing and Grass, 2003), which makes prokaryotic species highly sensitive to environmental copper variation.

Besides copper importers there are also copper efflux proteins, copper ATPases, which transport copper into the Golgi complex or into the extracellular space (Arguello et al., 2007, Thever and Saier, 2009). There are two copper ATPases in mammals expressed mainly in liver (ATP7B) and brain (ATP7A) and known also as Wilson's and Menkes' proteins in humans according to the diseases caused by their malfunction (Mercer, 2001).

A special class of proteins named copper chaperones has evolved for copper handling and delivery inside eukaryotic cells. Copper chaperones regulate copper homeostasis and trafficking of copper ions to the correct destination (Fig. 1) (Camakaris et al., 1999, Lutsenko, 2010, Robinson and Winge, 2010). Special copper chaperones have evolved for both intracellular copper enzymes, SOD1 and CCO. Metalation of SOD1 is accomplished by only one copper chaperone, called CCS. However, this chaperone does not only deliver the copper ion to SOD1, but it also catalyzes the formation of a critical disulfide bond within this enzyme, which is crucial for its function (Furukawa et al., 2004). Copper insertion into CCO in the mitochondrial IMS is an extremely complex process, which is mediated by at least six copper chaperone proteins, named Cox11, Cox17, Cox19, Cox23, Sco1 and Sco2 (Horng et al., 2004, Carr et al., 2005, Horn and Barrientos, 2008). A specific copper chaperone for Cu-ATPases is also present in eukaryotic cells. In yeast, this pathway involves a chaperone called Atx1 (Pufahl et al., 1997). Atx1 is very similar to CopZ, present in various bacteria (Odermatt and Solioz, 1995, Zhou et al., 2008). The homologue of Atx1 in mammals is called HAH1 (or Atox1) (Klomp et al., 1997).

Last but not least, all eukaryotic cells also contain small Cys-rich proteins called metallothioneins (MTs), which can bind several Cu(I) ions with high affinity and participate in copper buffering and regulation (Kagi and Schaffer, 1988, Palmiter, 1998).

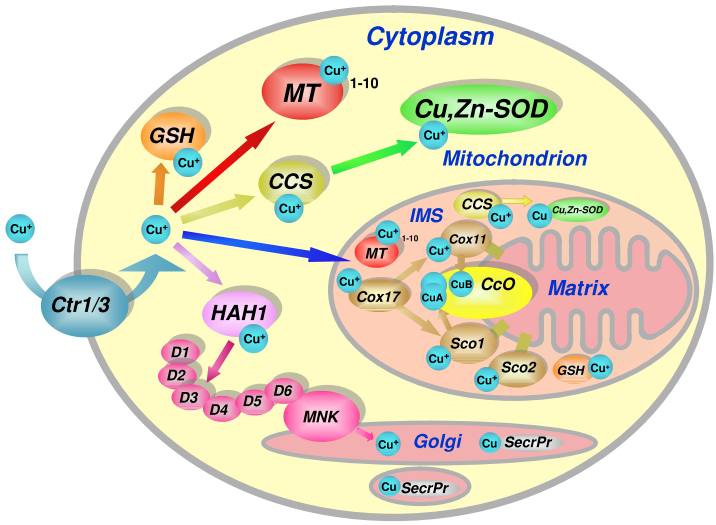

Figure 1. Copper trafficking pathways in a eukaryotic cell. Blue spheres – Cu ions; Ctr1/3 – copper influx transporters; CCS – copper chaperone for SOD1; HAH1 – copper chaperone for the ATP7A or Menkes' protein (MNK); D1–D6 – Cu(I)-binding domains of MNK; MT – metallothionein; GSH – glutathione; Cox11, Cox17, Sco1, Sco2 – copper chaperones and co-chaperones for cytochrome c oxidase; CCO - cytochrome c oxidase; SecPr – secreted copper proteins, such as ceruloplasmin, lysyl oxidase, tyrosinase and dopamine β-hydroxylase. Copper ions are delivered from Cox17 to Sco1 and/or Sco2, and to Cox11, which metalate the CuA and CuB sites of CCO, respectively.

1.2. Cytochrome c oxidase

Mitochondrial cytochrome c oxidase (CCO) is the final electron acceptor in the mitochondrial electron transport chain. This enzyme catalyzes electron transfer from cytochrome c to molecular oxygen, coupled with proton pumping from the mitochondrial matrix to the IMS and the generation of the transmembrane proton gradient, which is subsequently used by mitochondrial ATP synthase to drive the synthesis of ATP (Fig. 2) (Khalimonchuk and Rodel, 2005, Horn and Barrientos, 2008, Banci et al., 2010).

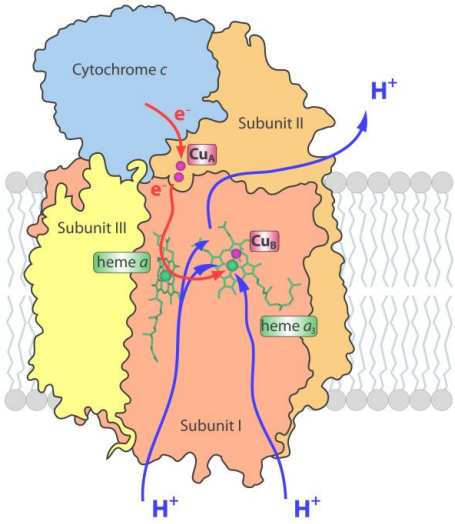

Figure 2. A schematic representation of cytochrome c oxidase subunits I, II, and III with the substrate cytochrome c. The routes of electron transfer (red arrows) and proton transfer (blue arrows) are shown. Copper ions are shown in two copper centers as purple spheres. Two heme centers are shown in green.

In bacteria the CCO complex resides in the cellular membrane and consists of only three major subunits that are highly conserved throughout evolution (Saraste, 1990). In eukaryotes the CCO complex resides in the inner mitochondrial membrane with portions protruding into the IMS and the matrix. The mitochondrial CCO complexes of yeast and mammals consist of 12 and 13 subunits, respectively (Yoshikawa et al., 1998). The three largest subunits, Cox1, Cox2 and Cox3, form the catalytic core of the enzyme (Fig. 2) and represent 60% of the mass of the enzyme (Carr and Winge, 2003, Herrmann and Funes, 2005). These subunits are encoded by mitochondrial DNA, whereas the remaining subunits are encoded by nuclear genes (Taanman, 1997, Ludwig et al., 2001). The nuclear encoded accessory subunits of CCO show low sequence conservation among eukaryotic species and they most likely have been acquired during the course of evolution (Fontanesi et al., 2006). The function of these accessory subunits is not well understood. It has been suggested that they may be involved in stabilization and regulation of the complex and in physical interactions with other enzymes of the respiratory chain (Fontanesi et al., 2006). The crystal structure of bovine CCO allows a detailed insight into the three-dimensional organization of the complex (Tsukihara et al., 1996, Tsukihara et al., 2003).

CCO contains four redox-active metal centers, which all participate in reducing molecular oxygen to water: two copper centers, Cu_A and Cu_B and two iron centers, heme a and heme a_3. Cu_B and two iron centers are located in subunit 1 (Cox1) and the dinuclear Cu_A center is located in subunit 2 (Cox2) (Fig. 2) (Tsukihara et al., 1995, Tsukihara et al., 2003). Electrons from cytochrome c enter CCO through a

mixed valence dinuclear Cu_A center that transfers electrons to heme a. From heme a the electrons are transferred intramolecularly to the active site composed of Cu_B and heme a_3, where oxygen binding occurs (Fig. 2). The mechanism of electron transfer in CCO has been extensively studied and several comprehensive reviews can be found on this subject (Hill, 1994, Brunori et al., 1998, Brunori et al., 2005, Brzezinski and Gennis, 2008, Abriata et al., 2008). The Cu_A site distinguishes this enzyme from other terminal oxidases that use quinol instead of cytochrome c as the electron donor (Hill, 1994, Babcock and Wikstrom, 1992, Saraste, 1994, Musser and Chan, 1998).

The assembly of eukaryotic CCO is an extremely complicated process occurring with the involvement of over 30 accessory proteins (Carr and Winge, 2003, Chinenov, 2000, Bertini and Cavallaro, 2008). The nuclear-encoded subunits must be imported to the mitochondrion through protein channels. Several processing steps are necessary for maturation of the core subunits and all of these processes are carried out with the assistance of specific proteins. Copper metalation of CCO subunits 1 and 2 is an essential step during the assembly of CCO. At least 6 copper chaperones – Cox17, Cox11, Cox19, Cox23, Sco1 and Sco2 – have been identified as key players in the delivery and insertion of copper ions into eukaryotic CCO (Carr and Winge, 2003, Cobine et al., 2006, Banci et al., 2008a). In the absence of copper centers, as well as in the absence of heme prosthetic groups, CCO proteins are rapidly degraded and the CCO holoenzyme fails to assemble (Barrientos et al., 2002, Stiburek et al., 2006).

Eukaryotic CCO also binds zinc and magnesium. The zinc ion, located in the noncatalytic subunit, can play a structural role (Coyne et al., 2007), whereas the magnesium/manganese site, located in close proximity to the H_2O exit channel, participates in the stabilization and release of H_2O produced as the result of the reduction of O_2 (Schmidt et al., 2003).

In recent years, biogenesis of the CCO complex has attracted much interest because defects in assembly of the enzyme are a major cause of mitochondrial disorders in humans and may also play an important role in aging and neurodegenerative diseases. Defective CCO biogenesis results in mitochondrial diseases frequently involving the brain, skeletal muscle, and heart (Schon, 2000, Shoubridge, 2001, Smeitink et al., 2001, Pecina et al., 2004, Rossi et al., 2004).

1.2.1. Copper chaperones for cytochrome c oxidase

Cox17, Cox19, Cox23, Cox11, Sco1 and Sco2 are the proteins involved in the metalation of CCO, and their knockout from yeast or other eukaryotic cells results in nonmetalated CCO (Tzagoloff et al., 1990, Horng et al., 2004, Horn and Barrientos, 2008, Khalimonchuk and Winge, 2008).

Table 1. Chaperones involved in CCO copper metalation.

Copper chaperone	Function	References
Cox17	Delivers copper to Sco1 and Cox11	(Glerum et al., 1996a, Beers et al., 1997, Banci et al., 2008b, Abajian et al., 2004)
Cox19	Copper trafficking in the IMS	(Nobrega et al., 2002, Barros et al., 2004)
Cox23		
Sco1	Copper metalation of Cu_A site of CCO	(Beers et al., 2002, Banci et al., 2006a)
Sco2	Copper metalation of Cu_A site of CCO	(Beers et al., 2002, Banci et al., 2007a)
Cox11	Copper metalation of Cu_B site of CCO	(Abajian et al., 2004, Banci et al., 2004a, Hiser et al., 2000, Carr et al., 2002)

Cox17

The importance of Cox17 is implied by early embryonic lethality of mice lacking Cox17 (Takahashi et al., 2002) at a stage similar to that of mice lacking the copper transporter Ctr1 (Lee et al., 2001). Cox17 is localized in both the cytosol and the intermembrane space of mitochondria (Glerum et al., 1996a, Beers et al., 1997). This small (~8 kDa) hydrophilic protein contains six conserved Cys residues and a metal-binding motif composed of two vicinal Cys residues (Abajian et al., 2004, Banci et al., 2008b). Two CX9C structural motifs of Cox17 form helical hairpin configurations, also referred to as CHCH (coiled coil-helix - coiled coil-helix) motifs (Arnesano et al., 2005, Banci et al., 2008b, Banci et al., 2009). The Cys residues within the twin CX9C motif form two disulfides, stabilizing the helical hairpin of Cox17 (Fig. 3) (Abajian et al., 2004, Arnesano et al., 2005).

Cox17 transfers copper ions to the CCO copper chaperones Sco1 (Glerum et al., 1996b, Leary et al., 2004) and Cox11 (Horng et al., 2004, Hiser et al., 2000, Carr et al., 2002, Horn and Barrientos, 2008). These proteins are anchored to the mitochondrial inner membrane through a transmembrane α-helix and expose their copper-binding sides in the IMS, where copper transfer occurs (Beers et al., 2002, Carr et al., 2005). Cox17 does not have a stable interaction with either Sco1 or Cox11 and it appears to use distinct interfaces to transfer Cu(I) to each target protein (Horng et al., 2004).

The yeast and mammalian Cox17 homologues share six conserved Cys residues involved in redox reactions as well as in metal binding and transfer (Arnesano et al., 2005). Cox17 exists in three oxidative states, each characterized by distinct metal-binding properties. Studies of mammalian Cox17 demonstrated that fully reduced Cox17(0S-S) cooperatively binds four Cu(I) ions (Palumaa et al., 2004).

Due to its low redox potential values, the fully reduced form is the most stable form of the protein in the cytoplasm (Voronova et al., 2007). Cox17(2S-S), containing two disulfide bridges, can bind one Cu(I) ion, whereas fully oxidized Cox17(3S-S), with three disulfide bridges, does not bind any metal ions (Palumaa et al., 2004). In the IMS, the protein is probably present mostly in the Cox17(2S-S) form (Voronova et al., 2007), which enables retention of the protein in the IMS and is able to transfer copper to Sco1 (Banci et al., 2007b).

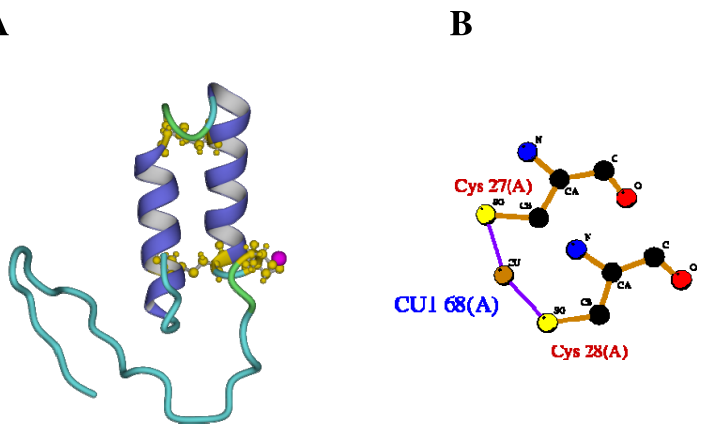

Figure 3. Solution structure of partially oxidized human Cu(I)Cox17(2S-S) (PDB code: 2RNB) (Banci et al., 2008b). The Cox17(2S-S) structure is stabilized by two disulfide bonds between the helices formed between two Cys pairs (yellow). Two additional Cys residues (yellow) coordinate the Cu(I) ion (purple sphere). The Yasara modeling program was used for visualization (Krieger et al., 2002) (A). Cu(I) ion coordination by two vicinal Cys residues. LIGPLOT presentation (Wallace et al., 1995) (B).

Cu(I) binding to Cox17(2S-S) occurs through two Cys residues in the vicinity of each other (Fig. 3) (Abajian et al., 2004, Arnesano et al., 2005, Banci et al., 2008b). The Cu(I) ion in CuCox17(2S-S) is partially solvent-exposed and Cu-reconstituted human Cox17 shows an S-Cu-S angle of 130° (Banci et al., 2008b). The bent coordination may permit an exogenous thiolate to provide a third ligand. The bent coordination of Cu(I) by the vicinal Cys residues in Cox17 reduces the binding affinity that may be required to permit the transfer of Cu(I) to Sco1 or Cox11 (Robinson and Winge, 2010).

Sco1 and Sco2

Two homologues of Sco proteins, Sco1 and Sco2, are expressed in yeast (Smits et al., 1994, Glerum et al., 1996b) as well as in mammals (Petruzzella et al., 1998, Leary et al., 2004). The Sco1 protein was first implicated in the metalation of CCO

since its overexpression rescued respiratory deficiency in yeast mutants lacking Cox17 (Glerum et al., 1996b). Yeasts lacking Sco1 are devoid of CCO activity and show greatly attenuated Cox2 protein levels (Schulze and Rodel, 1988, Krummeck and Rodel, 1990).

Sco1 and Sco2 are anchored to the inner mitochondrial membrane by a single functionally important transmembrane helix (Beers et al., 2002, Glerum et al., 1996b). A globular domain of Sco exhibiting a thioredoxin fold protrudes into the IMS (Nittis et al., 2001, Banci et al., 2007a). The "thioredoxin domain" of human Sco1 and Sco2 contains two essential Cys residues in a fully conserved CX3C metal-binding motif analogous to the copper-binding motif of Cox2 (Banci et al., 2006a, Banci et al., 2007a, Williams et al., 2005). The single Cu(I)-binding site is located within the globular domain of Sco1 and Sco2, consisting of two Cys residues within a CX3C motif and a conserved His residue (Fig. 4) (Banci et al., 2006a). Mutation of the Cys or His residues abrogates Cu(I) binding and leads to a nonfunctional CCO complex (Rentzsch et al., 1999, Nittis et al., 2001).

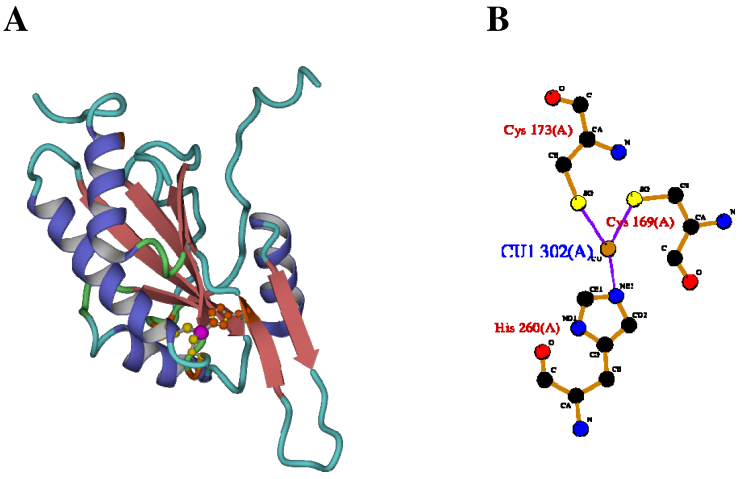

Figure 4. Solution structure of human Cu(I)Sco1 (PDB code: 2GQM) (Banci et al., 2006a). Helices are visualized in blue, β-sheets in red, Cys residues in yellow and the copper ion as a purple sphere. The Yasara modeling program was used for visualization (Krieger et al., 2002) (A). The Cu(I) ion is coordinated by two Cys and one His residue. LIGPLOT presentation (Wallace et al., 1995) (B).

The Cu(I) ion in Sco1 is partially solvent-exposed and ready for ligand exchange and transfer of the metal ion. The structures of metal-free human Sco1 and the Cu_1Sco1 complex are similar: only a single loop shows significant rearrangement

(Banci et al., 2006a). The movement of this loop positions the Cu(I)-binding His residue into the proper orientation for metal binding. Sco proteins can also bind Cu(II) (Horng et al., 2005, Andruzzi et al., 2005). It is not clear whether or not Sco1 transfers both Cu(I) and Cu(II) ions to build a binuclear Cu_A site with mixed valence in Cox2. Human Sco2 resembles human Sco1, although Sco2 shows greater conformational dynamics than Sco1 (Banci et al., 2007a). It remains unclear how exactly human Sco2 participates in Cu(I) transfer reactions during CCO assembly (Khalimonchuk and Winge, 2008).

Yeast Sco1 has been shown to interact with CCO, mediating copper transfer from Cox17 to the Cu_A site in Cox2 (Lode et al., 2000). The mechanism of interaction of Sco1 with both its upstream and downstream partners in the copper transfer pathway have been modeled *in silico* for both the Cox17–Sco1 and the Sco1–Cox2 adducts (van Dijk et al., 2007). It has been shown that Cox17–Sco1 interaction preferentially results from the partially oxidized Cox17(2S-S) form, and that a conserved Pro residue in Sco1 is necessary for the efficiency of copper transfer (Banci et al., 2007b).

NMR and X-ray studies of human Sco1 allowed Banci and coworkers to propose a model for Sco1–Cox2 copper transfer, involving the formation of a transient Sco1 species carrying a disulfide moiety able to interact with the copper ion (Banci et al., 2006a). The Cu_A center of Cox2 contains one Cu(I) and one Cu(II) ion, but it remains to be elucidated if Sco1 mediates the transfer of the ions in different oxidation states or, alternatively, if a Cu(I) ion inserted in Cox2 by Sco1 is successively oxidized. On the basis of the structural similarity of Sco 1 and 2 to the family of disulfide reductases it has been suggested that Sco proteins may be involved in the reduction of Cys in the Cox2 copper-binding site (Chinenov, 2000, Balatri et al., 2003, McEwan et al., 2002, Williams et al., 2005, Abajian and Rosenzweig, 2006). This hypothesis is supported by the results of NMR studies (Banci et al., 2006a) demonstrating that human Cox17(2S-S) can reduce the disulfide in oxidized human Sco1 protein but not in human Sco2, and thus it can distinguish between the two proteins with similar Cu(I)-binding affinities and selectively metalate human Sco1 (Banci et al., 2008a). The reduction is necessary for copper incorporation (Abajian and Rosenzweig, 2006, Ye et al., 2005). A similar reaction where Sco1 acts as a thioredoxin can also occur when Cu(I)Sco1 transfers a copper ion to the Cu_A site in CCO (Banci et al., 2006a). Oxidoreductase activity has also been observed in Sco1 of *T. thermophilus (Tt). Tt* Sco1 reduced the disulfide bond in the active center of Cu_A, allowing another protein to insert Cu(I) ions into CCO (Abriata et al., 2008). Thus, mammalian Sco1 may function as a redox switch, in which the oxidation of the two Cys residues in the Cu(I)-binding CX3C motif may facilitate Cu(I) transfer to Cox2 (Balatri et al., 2003).

The biological function of Sco proteins is still an unresolved issue (Khalimonchuk and Winge, 2008). In yeast, only Sco1 is required for CCO assembly, and the biological role of Sco2 is unclear (Glerum et al., 1996b), whereas in humans both Sco1 and Sco2 are essential for the process (Papadopoulou et al., 1999, Jaksch et al., 2000, Sue et al., 2000, Shoubridge, 2001). Genetic and biochemical analyses of Sco1 and Sco2 patient cell lines have suggested that the two human proteins have independent, cooperative functions in CCO assembly (Leary et al., 2004), and regulatory roles in the maintenance of cellular copper homeostasis (Leary et al., 2007, Briere and Tzagoloff, 2007). In humans Sco2 is implicated in a redox function (Leary et al., 2009). It has also been suggested that human Sco2 can form a heterodimer with Sco1 and that this dimer could be needed for metalation of the Cu(II)-Cu(I) site in Cu_A (Horng et al., 2005, Horn and Barrientos, 2008).

Yeast Sco2 apparently has different functions from those of the human homologue, as deletion of Sco2 does not affect CCO assembly (Glerum et al., 1996b, Papadopoulou et al., 1999, Jaksch et al., 2000). However, Sco2 overexpression can partially rescue a Sco1 point mutant (Glerum et al., 1996b). In addition, Sco2 overexpression suppresses the effects of Cox17 mutations, although it does this less efficiently than Sco1 (Glerum et al., 1996b). These data indicate that yeast Sco1 and Sco2 have overlapping but not identical functions (Glerum et al., 1996b).

Mutations in either human Sco1 or Sco2 lead to decreased CCO activity and early death. Patients with mutations in Sco2 have a clinical presentation distinct from that of patients having mutations in Sco1 (Papadopoulou et al., 1999, Jaksch et al., 2000, Valnot et al., 2000, Shoubridge, 2001). Patients with mutated Sco2 have neonatal encephalopathy and cardiomyopathy (Jaksch et al., 2000, Papadopoulou et al., 1999), whereas patients with mutated Sco1 exhibit neonatal hepatic failure (Valnot et al., 2000). The distinctive clinical presentation is not a result of tissue-specific expression of the two genes, as Sco1 and Sco2 are ubiquitously expressed and exhibit a similar expression pattern in different human tissues (Leary et al., 2004).

Cox11

Cox11 is the metallochaperone that assists in the formation of the Cu_B site of CCO (Hiser et al., 2000, Carr et al., 2002). The Cu_B site in the Cox1 subunit of CCO is formed by one copper ion coordinated by three His ligands in close proximity to the heme a_3 (Hiser et al., 2000). The Cox11 monomer binds a single Cu(I) ion and coordinates it via three thiolate ligands. Mutation of any of these Cys residues reduces Cu(I) binding and confers respiratory incompetence (Carr et al., 2002). Yeast *S. cerevisiae* cells lacking Cox11 have impaired CCO activity and lower levels of Cox1 (Tzagoloff et al., 1990). Similarly to Sco1, Cox11 has one transmembrane domain and a soluble copper-binding domain which faces the IMS (Carr et al., 2002, Carr et al., 2005, Khalimonchuk and Rodel, 2005). It has been

shown that the matrix domain of Cox11 is not essential for its function (Carr et al., 2005).

The structure of Cox11 was solved in 2004 (Banci et al., 2004a). However, it is still unclear how Cox11 interacts with Cox17 and with CCO and how the transfer of copper ions to the Cu_B site is mediated. It has been suggested that copper transfer from Cox11 to the Cu_B site, deeply buried inside the mitochondrial inner membrane, takes place during the insertion and folding of nascent Cox1 chains within the inner membrane (Khalimonchuk and Rodel, 2005, Carr and Winge, 2003). This assumption is supported by the observation of weak interaction of Cox11 with the mitochondrial ribosome (Khalimonchuk et al., 2005). As the Cu_B site is a heterobimetallic site with heme a_3, formation of the Cu_B site is likely to be concurrent with heme a_3 insertion occurring prior to the addition of Cox2 and Cox3 subunits into the CCO complex (Smith et al., 2005). Computer models suggest direct interaction between Cox11 and Cox1 (Khalimonchuk et al., 2007), an interaction that would serve not only in copper transfer to the Cu_B site but also in protection of early Cox1-heme complex against oxidative damage (Khalimonchuk et al., 2007).

Cox19 and Cox23

The IMS contains two small polypeptides, containing a twin CX9C motif present in Cox17, which are also relevant for CCO assembly. These proteins are Cox19 and Cox23 (Nobrega et al., 2002, Barros et al., 2004). The recombinant form of Cox19 binds copper (Rigby et al., 2007) and the CCO assembly defect of a *cox19* null mutant strain could not be rescued by copper supplementation to the media (Nobrega et al., 2002). Mutants of Cox23 also fail to assemble CCO but their respiratory-deficient phenotype is complemented by exogenous copper supplementation, although only with concomitant overexpression of Cox17 (Barros et al., 2004). Cox23 does not physically interact with Cox17 in the form of a stable complex. It has been shown that Cox23 is required for mitochondrial copper homeostasis, where it functions in a common pathway with Cox17 (Barros et al., 2004).

Copper metalation of CCO requires the participation of several metallochaperone complexes. The list of mitochondrial proteins that affect mitochondrial copper homeostasis and CCO assembly is steadily growing. How mitochondrial copper metabolism affects cellular copper homeostasis is one of the remaining questions to be answered in this field. Understanding the fundamental aspects of mitochondrial copper metabolism and CCO assembly will help to understand the function of enzymes crucial for aerobic production of energy. This will also contribute to a better understanding of human mitochondrial diseases resulting from mutations in the genes and pathways involved in copper homeostasis (Horn and Barrientos, 2008).

1.3. Cu,Zn-superoxide dismutase

Cu,Zn-superoxide dismutase (SOD1) represents the major cellular defense system against oxidative damage (McCord and Fridovich, 1969). SOD1 uses the redox properties of copper to catalyze the disproportionation of superoxide anion to oxygen and hydrogen peroxide, thereby protecting cells against oxidative damage (McCord and Fridovich, 1969);

$Cu^{2+},Zn^{2+}\text{–SOD1} + O_2^- \leftrightarrow Cu^{1+},Zn^{2+}\text{–SOD1} + O_2$
$Cu^{1+},Zn^{2+}\text{–SOD1} + O_2^- + 2H^+ \leftrightarrow Cu^{2+},Zn^{2+}\text{–SOD1} + H_2O_2$

The major source of O_2^- is the respiration process in mitochondria: approximately 1–2% of daily consumed oxygen is converted to O_2^- in mammals (Cadenas and Davies, 2000). Eukaryotic SOD1 is mainly localized in cytosol with a smaller fraction present in the IMS of mitochondria (Field et al., 2003, Lindenau et al., 2000, Sturtz et al., 2001, Crapo et al., 1992). However, it has also been found in nuclei, lysosomes and peroxisomes (Chang et al., 1988). The intracellular concentration of SOD1 is considerably high, ranging from 10 to 100 μM (Kurobe et al., 1990, Lindenau et al., 2000). This level is sufficient to ensure the conversion of physiological amounts of superoxide radicals. In the mitochondrial matrix the dismutation reaction is carried out by mitochondrial Mn-SOD (SOD2) (Weisiger and Fridovich, 1973, Tyler, 1975). There is no sequence or structural similarity between the Cu,Zn-SOD and Mn,Fe-SOD enzyme families. Extracellular SOD (SOD3), which belongs to the Cu,Zn-SOD family, diverged from cytosolic Cu,Zn-SOD (SOD1) at early stages of evolution before the differentiation of fungi, plants and metazoa (Bordo et al., 1994, Zelko et al., 2002). Members of these families are found in all living organisms: prokaryotes, archaea and eukaryotes (Culotta et al., 2006).

SOD1 is a 32 kDa homodimeric enzyme with a highly conserved sequence, present in prokaryotes and eukaryotes (Bordo et al., 1994). SOD1 subunits have an immunoglobulin-like β-sandwich fold with an intrasubunit disulfide bond (Khare et al., 2004). The intramolecular disulfide bond in SOD1 is conserved in SOD1 proteins from all species studied (Abernethy et al., 1974, Beyer et al., 1987), and this disulfide bond is essential for enzymatic activity. An important feature of the SOD1 disulfide bond is its high stability in the cytosolic reducing environment (Forman and Fridovich, 1973, Furukawa et al., 2004, Arnesano et al., 2004). In the absence of metal cofactors the disulfide bond is reduced and SOD1 exists in the form of an inactive monomer (Furukawa et al., 2004, Forman and Fridovich, 1973).

Activation of SOD1 requires the binding of both zinc and copper ions in each subunit (Fig. 5). Copper is involved in catalysis whereas zinc is not essential for the dismutation activity, but it ensures the high stability of active SOD1 (Furukawa et al., 2004, Potter et al., 2007).

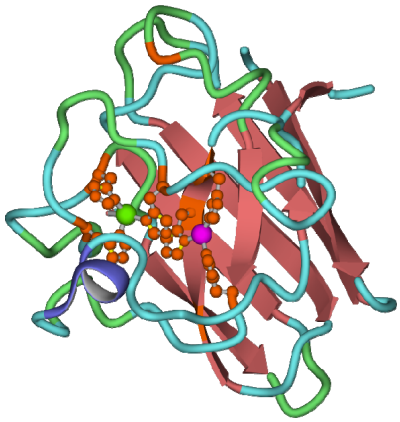

Figure 5. Solution structure of reduced monomeric copper- and zinc-bound SOD1 (PDB code: 1BA9) (Banci et al., 1998). A helix is visualized in blue, β-sheets in red, coordinating residues in orange, copper as a purple sphere and zinc as a green sphere. The Yasara modeling program was used for visualization (Krieger et al., 2002).

A Cu(I) ion is coordinated by three His residues, and binds water as an additional ligand (Banci et al., 1998) (Fig. 6).

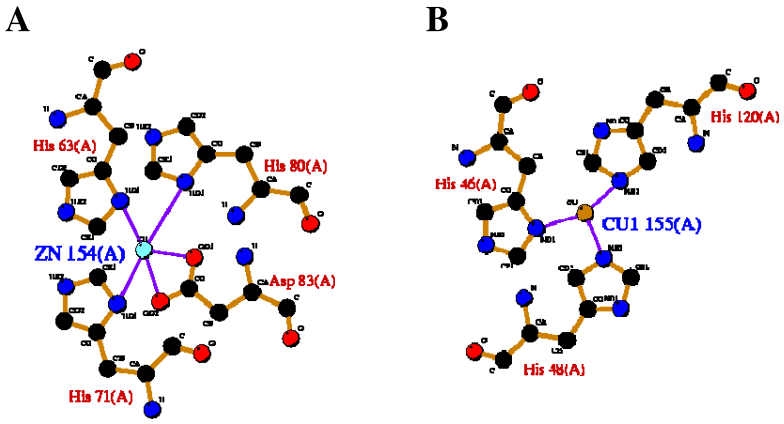

Figure 6. Metal ion coordination in human reduced monomeric copper- and zinc-bound SOD1 (PDB code: 1BA9) (Banci et al., 1998). The zinc ion is coordinated by three His and one Asp residue (A). The Cu(I) ion is coordinated by three His residues (B). LIGPLOT presentation (Wallace et al., 1995).

SOD1 is expressed in many tissues with higher levels in liver and kidney (Asayama and Burr, 1985). It is also abundant in motor neurons (Pardo et al., 1995). Knockout studies indicate that elimination of *sod1* results in widespread oxidative damage (Elchuri et al., 2005). Reduction of SOD1 activity may lead to an altered redox status in diabetic patients (Haskins et al., 2004). Over 100 different point mutations in SOD1 have been reported to cause the familial form of amyotrophic lateral sclerosis (ALS) (Bruijn et al., 2004). Many of the mutant proteins exhibit normal SOD1 activity, although they have an increased tendency for aggregation and amyloidogenesis. This indicates that some aberrant 'gain-of-function' by SOD1 is associated with the neurodegeneration observed in ALS patients (Rakhit et al., 2002).

1.3.1. Copper chaperone for superoxide dismutase – CCS

The delivery of copper to SOD1 requires a special copper chaperone, called CCS (Culotta et al., 1997, Casareno et al., 1998, Rae et al., 1999, Schmidt et al., 2000, Rae et al., 2001). CCS induces the activation of monomeric reduced SOD1, facilitating both Cu(I) transfer and disulfide bond formation between Cys57 and Cys146 (Furukawa et al., 2004, Culotta et al., 2006).

The copper chaperone CCS was first identified in the yeast strain *S. cerevisiae* (Culotta et al., 1997) and it has been found to be an important regulator of SOD1 structure and function. SOD1 activation in yeast cells is strictly dependent upon the existence of CCS (Schmidt et al., 1999, Rae et al., 1999). CCS proteins have been identified and studied in various species including humans (Casareno et al., 1998), rodents (Wong et al., 2000), insects (Southon et al., 2004), and plants (Wintz and Vulpe, 2002). CCS-knockout mice and CCS−/− mouse fibroblast cells have been shown to retain a certain degree of SOD1 activity, suggesting the presence of an alternative CCS-independent pathway of SOD1 activation in mammalian cells (Subramaniam et al., 2002, Carroll et al., 2004). The nematode *C. elegans* does not have any CCS homologue and thus this organism relies on a CCS-independent pathway for SOD1 activation (Jensen and Culotta, 2005). Human SOD1 can also be partially activated independently of CCS when expressed in yeast or flies (Carroll et al., 2004, Kirby et al., 2008). It has been shown that two Pro residues in the C-terminus of yeast SOD1 hinder the CCS-independent activation of SOD1. Replacement of these Pro residues restores and insertion abrogates the CCS-independent activation (Carroll et al., 2004). CCS levels are significantly elevated under conditions of copper deficiency (Bertinato et al., 2003, West and Prohaska, 2004).

CCS is highly selective; yeast Cu-CCS can only activate reduced yeast apo-SOD1 in its monomeric form, and dimeric apo-SOD1, which already possesses an intramolecular disulfide bond, cannot be metalated by the chaperone (Furukawa et al., 2004). In 2004 it was shown that CCS can activate apo-SOD1 only in the presence of oxygen, which is required for disulfide formation in SOD1 (Brown et al., 2004, Furukawa and O'Halloran, 2006). Under anaerobic conditions the Cys residues in yeast SOD1 remain reduced even after incubation with Cu-CCS and the protein remains inactive. Expression and activation of SOD1 is triggered within an hour after oxygen exposure (Brown et al., 2004). CCS is also the key activator of SOD1 within the mitochondrial IMS, where 1% to 5% of SOD1 resides. It has been shown that reduced apo-SOD1 is imported from cytosol into the IMS as an unfolded protein (Field et al., 2003, Arnesano et al., 2004). The presence of SOD1 within the IMS is largely dependent on CCS: in the absence of CCS, only minimal levels of SOD1 are observed in the IMS (Sturtz et al., 2001, Field et al., 2003).

CCS is a 28 kDa protein comprised of three domains (Fig. 7) (Schmidt et al., 1999:Schmidt, 2000 #550). Domains 1 and 3 bind Cu(I), whereas domain 2 is the key domain for interaction with SOD1 (Fig. 7) (Lamb et al., 2000, Lamb et al., 2001, Schmidt et al., 1999).

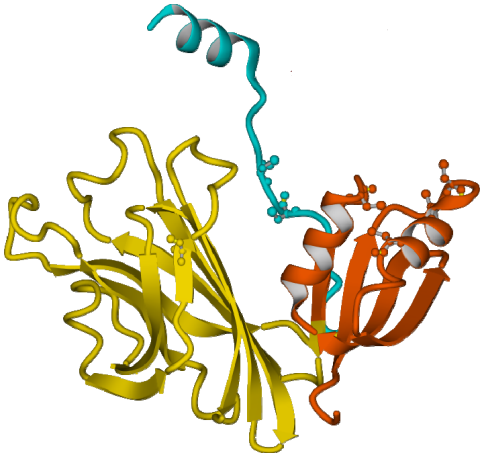

Figure 7. Crystal structure of CCS (PDB code: 1JK9) (Lamb et al., 2001). CCS domain 1 is shown in red, domain 2 in yellow, and domain 3 in blue. The side chains of the Cys residues are shown. The Yasara modeling program was used for visualization (Krieger et al., 2002).

Domain 1 (D1) of CCS (~8kDa) contains a βαββαβ-fold (Fig. 7) and is able to bind a single Cu(I) ion (Stasser et al., 2007). Two Cys residues in the MXCXXC motif of CCS D1 are involved in the copper binding (Eisses et al., 2000). D1 of yeast CCS is not essential for SOD1 activation in normal growth conditions, but it is required for SOD1 activation when intracellular availability of copper is limited (Schmidt et al., 1999). A human CCS mutant, with Cys substitutions in the

MXCXXC motif and Cu(I) bound to domain 3, is still able to activate SOD1, although to a lower extent than the full-length CCS (Stasser et al., 2007).

Domain 2 (D2) of CCS (~ 16 kDa) is highly homologous to SOD1. There is 47% sequence identity between human SOD1 and CCS D2. No copper-binding sites are found in CCS D2 (Schmidt et al., 1999), whereas D2 binds an equimolar amount of Zn(II) (Rae et al., 2001), which is most probably required for protein stabilization (Endo et al., 2000). It has been proposed that CCS interacts with SOD1 through D2 by mimicking SOD1 dimerization, since SOD1 with mutations at the dimerization interface is not activated by CCS (Schmidt et al., 2000). Similarly, when the corresponding amino acid residues in yeast CCS D2 are mutated, activation of SOD1 is not observed (Schmidt et al., 2000).

Domain 3 (D3) of CCS is a short polypeptide (30–40 amino acids) essential for the function of CCS. The CXC motif found in D3 is highly conserved among all species. CCS lacking the D3 CXC motif fails to activate SOD1 (Schmidt et al., 1999, Caruano-Yzermans et al., 2006, Stasser et al., 2005, Stasser et al., 2007).

The copper-binding site in SOD1 is buried in the protein interior. CCS docks with and transfers the metal ion to the reduced apoSOD1 (Rae et al., 2001). In the SOD1-CCS heterodimer, an intermolecular disulfide bond forms between Cys 57 (SOD1) and Cys 229 (CCS), and this disulfide is transformed into an intramolecular disulfide bond between Cys 57 and 146 in active SOD1. This finding implies the involvement of CCS in disulfide formation in SOD1 and highlights the essential role of this disulfide in the SOD1 activation mechanism. The copper transfer in the heterodimeric complex to SOD1 induces conformational changes that promote the conversion of intermolecular to intramolecular disulfide (Fig. 8) (Culotta et al., 2006, Furukawa and O'Halloran, 2006).

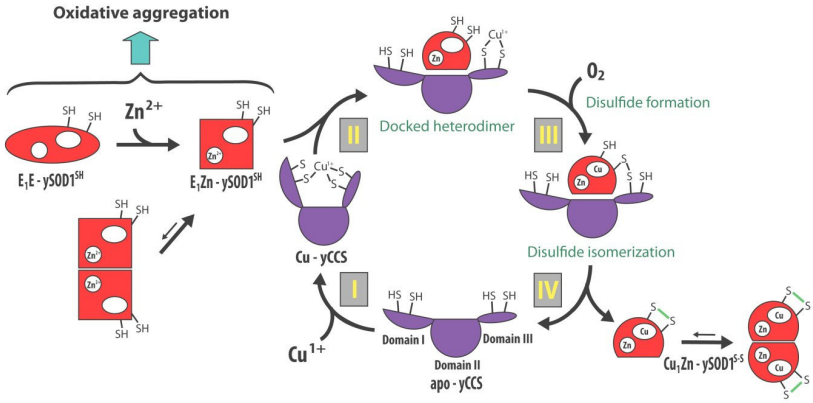

Figure 8. Proposed mechanism of SOD1 activation by its metallochaperone CCS according to Ref. (Culotta et al., 2006). The copper chaperone acquires copper through unknown routes and then docks with a reduced form of SOD1 (steps I and II). This complex is inert to further reaction unless exposed to oxygen or superoxide (step III), at which point a disulfide-linked heterodimeric intermediate forms. This complex undergoes disulfide exchange from intermolecular to an intramolecular disulfide in SOD1 (step IV). Copper is transferred at some point after introduction of oxygen and the mature monomer is proposed to be released from CCS. The left side of the image depicts several immature states of the protein in which the essential disulfide bond has not yet formed. Oxidation of the conserved Cys residues and the formation of incorrect disulfide linkages can lead to SOD crosslinking and aggregation.

1.4. Cu-ATPases

Organisms from all kingdoms of life employ P_{1B}-type ATPases to transport copper ions across membranes (Arguello et al., 2007). In prokaryotes, Cu(I) ATPases are used primarily to export excess copper from the cell, whereas in eukaryotes, Cu(I) ATPases also transport copper to the secretory pathway for incorporation into secretory copper enzymes (Arguello et al., 2007, Lutsenko et al., 2007a, Thever and Saier, 2009).

The N-terminal domains of copper trafficking ATPases from different organisms have similar structures, although they contain different numbers of intracellular metal binding domains (MBDs). Prokaryotic (Arguello, 2003, Banci et al., 2002) and yeast (Yuan et al., 1995) homologues contain only one or two MBDs, and *D. melanogaster* (Norgate et al., 2006), rat (Wu et al., 1994) and human homologues (Lutsenko et al., 2002, Voskoboinik et al., 1999) have four, five and six MBDs, respectively. The increase in the number of MBDs of copper-transporting ATPases

from bacteria to mammals is likely to be linked to the evolution of multicellular organisms, which must face the additional task of intercellular copper trafficking (Bertini and Cavallaro, 2008).

Humans have two Cu(I)-transporting ATPases: ATP7A is also called Menkes' disease protein and ATP7B is called Wilson's disease protein (Chelly et al., 1993, Vulpe et al., 1993, Bull et al., 1993, Tanzi et al., 1993). ATP7A and ATP7B are structurally homologous, but they are expressed in different tissues (Lutsenko et al., 2007a, Wernimont et al., 2000, Yatsunyk and Rosenzweig, 2007). The expression level of ATP7B is highest in the liver, whereas lower levels of the protein are detected in kidneys, heart, brain and muscles (Bull et al., 1993, Tanzi et al., 1993). ATP7A has a high expression level in intestinal mucosa, lungs, kidneys and neuronal cells but is not expressed in the liver (Vulpe et al., 1993, Monty et al., 2005, Veldhuis et al., 2009).

ATP7A and ATP7B are important regulators of copper homeostasis in human cells, and their general functions are very similar. The primary role of both proteins is connected with the transport of copper from the cytosol into the secretory pathway for further incorporation into copper-dependent enzymes. ATP7A and ATP7B also regulate the intracellular copper concentration by exporting excess copper from the cells, hence playing a dual role in human cells (Petris et al., 1996, Roelofsen et al., 2000, Goodyer et al., 1999, Forbes et al., 1999). Switching of roles occurs via relocalization of the protein depending on the cellular copper content. At basal copper concentrations, ATP7A and ATP7B are located in the *trans*-Golgi network (TGN), where they deliver copper to the secretory pathway (Nyasae et al., 2007, Veldhuis et al., 2009, Monty et al., 2005). When the copper content is elevated, ATP7A and ATP7B relocate to vesicles and eventually to the plasma membrane to export excess copper from the cell (Petris et al., 1996, Hung et al., 1997, Mercer et al., 2003, Veldhuis et al., 2009, Monty et al., 2005, Nyasae et al., 2007). It has been suggested that copper efflux is mediated by ATP7A and ATP7B accumulating copper in the vesicles, followed by vesicle fusion to the plasma membrane and exocytosis, rather than via direct transport of copper across the plasma membrane by ATP7A and ATP7B (Petris and Mercer, 1999, Schaefer et al., 1999, Monty et al., 2005, Lutsenko et al., 2007a, Veldhuis et al., 2009).

The ATP7A and ATP7B proteins share 69% similarity and consist of three putative regions: (i) a transmembrane (TM) region where eight helices form a channel for metal ion passage; (ii) an ATP-binding domain; and (iii) a cytosolic N-terminal metal-binding region with six copper-binding domains (Fig. 9) (Lutsenko et al., 2002, Arnesano et al., 2002, Banci et al., 2010). The intramembrane metal-binding site involves a conserved CPC motif in transmembrane helix 6 (TMH6) and several additional invariant residues in TMH7 and TMH8 (Axelsen and Palmgren, 1998, Arnesano et al., 2002, Arguello, 2003, Arguello et al., 2007). Mutations in the two

Cys residues of the CPC motif impair enzyme function (Mandal and Arguello, 2003).

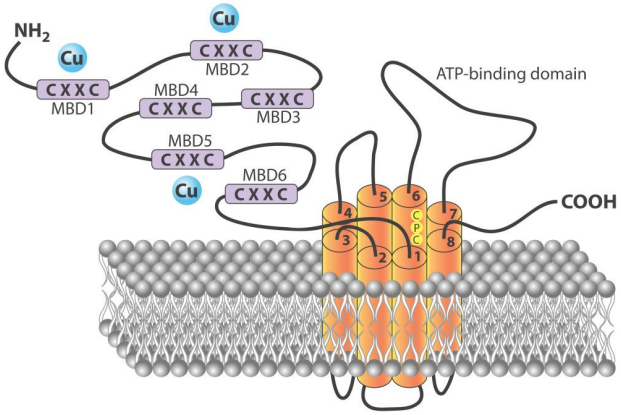

Figure 9. The copper-transporting P_{1B}-type ATPase. Proposed structure of the ATPase homologues ATP7A and ATP7B. Highlighted are six metal-binding domains (MBDs) in the amino terminus, each containing a CXXC copper-binding motif, the ATP-binding domain and the CPC copper-binding motif in the intracellular part of the ATPase.

The ATP-binding domain contains a nucleotide-binding site and an Asp residue in the invariant DKTGT sequence, which becomes phosphorylated during the ATP hydrolysis cycle (Voskoboinik et al., 2001, Tsivkovskii et al., 2002, Boal and Rosenzweig, 2009, Banci et al., 2010). The cytosolic N-terminal metal-binding region of both ATP7A and ATP7B is composed of six homologous MBDs (Fig. 9). Each ~70-residue MBD exhibits a βαββαβ fold (Fig. 10) and contains a CXXC metal-binding motif (Achila et al., 2006, Banci et al., 2005b, Banci et al., 2004c, Gitschier et al., 1998). The two Cys residues in the CXXC motif bind one Cu(I) ion in a distorted linear fashion (Fig. 10) (DiDonato et al., 2000, DiDonato et al., 1997, Lutsenko et al., 1997, Ralle et al., 1998, Ralle et al., 2004, Banci et al., 2005b, Yatsunyk and Rosenzweig, 2007).

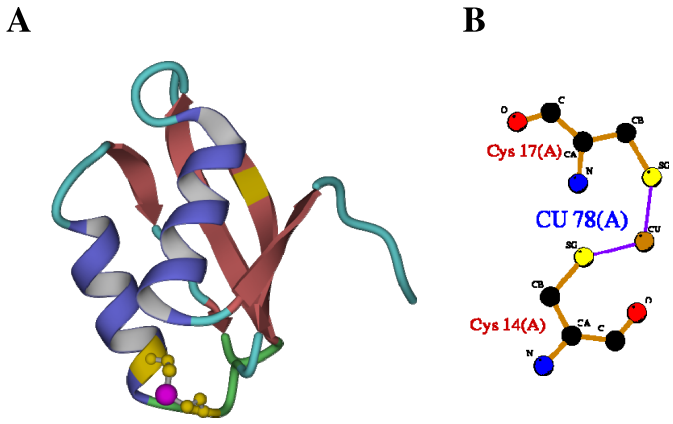

Figure 10. Solution structure of the Cu(I) bound fifth domain of human ATP7A (PDB code: 1Y3J) (Banci et al., 2005b). Helices are visualized in blue, β-sheets in red, Cys residues in yellow, and copper as a purple sphere. The Yasara modeling program was used for visualization (Krieger et al., 2002) (A). A Cu(I) ion is coordinated by two Cys residues. LIGPLOT presentation (Wallace et al., 1995) (B).

The role of multiple MBDs is not fully understood, since only one or two of them are required to ensure copper transport (Forbes et al., 1999, Mercer et al., 2003, Cater et al., 2004). The remaining four MBDs are suggested to be involved in copper acquisition when copper concentrations are low or to be sensors of the intracellular copper concentration that might participate in the regulation of ATPase activity or switching of protein localization from the *trans*-Golgi network to the basolateral (ATP7A) or apical (ATP7B) membrane (Voskoboinik et al., 2001, Tsivkovskii et al., 2001, Strausak et al., 1999, Nyasae et al., 2007, Veldhuis et al., 2009).

Mutations or deletions in genes encoding ATP7A or ATP7B proteins are associated with severe disorders of copper metabolism named Menkes' disease (MD) and Wilson's disease (WD) (Chelly et al., 1993, Bull et al., 1993, Mercer, 2001). The former is an X-linked lethal disorder (Menkes et al., 1962) resulting in copper accumulation in intestinal cells and an insufficient copper supply to distant organs and peripheral tissues (Danks et al., 1972, Madsen and Gitlin, 2007). The etiology of MD involves low concentrations of copper in plasma due to impaired intestinal absorption (Danks et al., 1972, Danks et al., 1973, Lutsenko and Petris, 2003, Lutsenko et al., 2007a), plus low levels of liver and brain copper (Kodama and Murata, 1999, Liu et al., 2005, Lutsenko et al., 2007a, Madsen and Gitlin, 2007). In contrast, certain other tissues (duodenal mucosa, kidney, spleen,

pancreas, skeletal muscle, and placenta) tend to accumulate copper in this disorder (Keydorn et al., 1975, Goka et al., 1976, Horn, 1981, Lutsenko et al., 2007a).

The gene responsible for WD was identified in 1993 (Tanzi et al., 1993, Bull et al., 1993). In WD the function of the ATP7B protein is lost, leading to a deficiency in copper transport to the secretory pathway and accumulation of copper in various organs. The disease is an autosomal recessive disorder of copper metabolism characterized by copper accumulation in the liver, central nervous system, kidney and other organs, leading to liver cirrhosis and neurological disorders (Pilloni et al., 1998, Faa et al., 1995, Kitzberger et al., 2005, Madsen and Gitlin, 2007, Crisponi et al., 2010, Bertini and Cavallaro, 2008). Wilson's disease was fatal until treatments for halting copper storage were developed in the fifties. In cases of WD, a chelation therapy, using different medications such as penicillamine, trien and tetrathiomolybdate, is applied. WD was the first chronic liver disease for which an effective pharmacologic treatment was discovered (Crisponi et al., 2010).

1.4.1. Copper chaperone for Cu-ATPases – HAH1 (Atox1)

Human P_{1B}-type ATPases acquire Cu(I) from a copper chaperone, HAH1, (Klomp et al., 1997, Hamza et al., 1999, Lutsenko et al., 2007a, Hamza et al., 2003, Walker et al., 2002). HAH1 is a small protein composed of 68 amino acid residues and it is present in cytoplasm and in the cell nucleus (Klomp et al., 1997). The family of HAH1-related copper chaperones is highly conserved in all organisms ranging from bacteria to higher eukaryotes (Arnesano et al., 2002). Homologues of HAH1 are found in cyanobacteria (Atx1) (Banci et al., 2004b), in *Bacillus subtilis* (CopZ) (Banci et al., 2001), in yeast (Atx1) (Lin et al., 1997, Pufahl et al., 1997), in humans (HAH1) (Klomp et al., 1997) and in many other organisms (Tottey et al., 2005). The majority of HAH1 homologues are ~70-amino-acid proteins containing a conserved metal-binding motif, CXXC. The same motif is also found in the MBDs of P_{1B}-ATPases (Rosenzweig, 2001, Arnesano et al., 2002).

The structures of HAH1 have been determined by X-ray and NMR methods (Boal and Rosenzweig, 2009) and the solution structures of apo and Cu(I)-HAH1 are very similar to the crystal structures (Anastassopoulou et al., 2004, Wernimont et al., 2000). Both prokaryotic and eukaryotic HAH1 homologues exhibit a $\beta\alpha\beta\beta\alpha\beta$ fold (Arnesano et al., 2001, Banci et al., 2001, Wimmer et al., 1999, Rosenzweig et al., 1999, Wernimont et al., 2000). However, the solution structure and the crystal structure of Cu(I)-HAH1 show clearly different metal coordination. In the crystal a single metal ion is coordinated by two protein molecules of the HAH1dimer, while in the solution structure the protein is monomeric and the copper ion is coordinated by two Cys residues of the same protein molecule (Anastassopoulou et al., 2004). The latter coordination geometry corresponds to that under physiological

conditions. The majority of conformational changes upon Cu(I) binding are localized to the metal-binding loop. After Cu(I) binding the positively charged side chain of the Lys60 residue shifts toward the metal-binding site, which may stabilize the overall negative charge on the copper ion in the Cu(I)-thiolate complex (Rosenzweig et al., 1999).

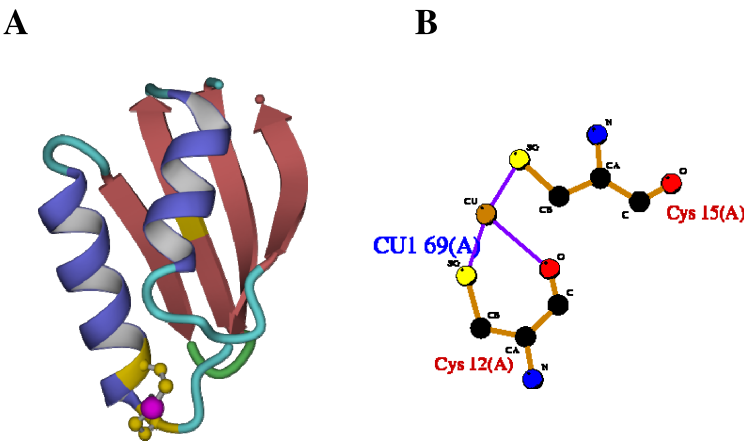

Figure 11. Solution structure of human Cu(I)HAH1 determined by NMR (PDB code: 1TL4) (Anastassopoulou et al., 2004). Helices are visualized in blue, β-sheets in red, Cys residues in yellow and copper as a purple sphere. The Yasara modeling program was used for visualization (Krieger et al., 2002) (A). Cu(I) ion coordination by two Cys residues. LIGPLOT presentation (Wallace et al., 1995) (B).

Atx1 is structurally similar to the MBDs of Cu-ATPases (Figs. 10 and 11), which allow the metallochaperone to bind to the ATPase, thereby facilitating the transfer of Cu(I) through ligand exchange reactions (Lamb et al., 2001, Banci et al., 2006b). Indeed, it has been shown that the MBDs of Cu-ATPases receive copper from the chaperone HAH1 via direct protein-protein interactions (Achila et al., 2006, Hamza et al., 1999, Strausak et al., 2003, Banci et al., 2005a). Different MBDs exhibit different interactions with HAH1, although domains two and/or four are the preferential copper acceptors (Achila et al., 2006).

It has been demonstrated that MBD6, the domain closest to the transmembrane region of Cu-ATPases, can directly receive copper from Cu(I)-HAH1 (Cater et al., 2004, Huster and Lutsenko, 2003). However, a mechanism by which Cu(I)-HAH1 initially forms an adduct with domains 1, 2 or 4, after which the Cu(I) ion is transferred to domain 6 or 5, has been also suggested (Achila et al., 2006). Copper

transfer from HAH1 to the MBDs ultimately leads to the movement of Cu(I) across the membrane of the secretory compartment. Though much effort has been made in understanding the catalytic mechanisms of Cu(I)-transporting ATPases (Lutsenko and Petris, 2003, Lutsenko et al., 2007b), it is not clear how Cu(I) is transferred from the N-terminal MBDs of the transporters to the transmembrane domain (Kim et al., 2008).

1.5. Metallothioneins

Metallothioneins (MTs) are a superfamily of low-molecular-mass Cys-rich proteins with high metal-binding capacities (Kagi and Schaffer, 1988). They are highly conserved through evolution and are present in all eukaryotes and in certain prokaryotes (Hamer, 1986, Coyle et al., 2002, Vallee, 1995, Palmiter, 1998). In mammals the MT gene family consists of four subfamilies designated MT-1 through MT-4 (Vasak and Hasler, 2000), whilst yeast encodes only two MTs (Cup1 and Crs5) (Culotta et al., 1994, Jensen et al., 1996). Only one member of each subfamily is present in mice, but there are 10 functional MT genes in humans (Miles et al., 2000). Mammalian MTs are composed of a single polypeptide chain of 61–68 amino acids with a conserved array of 20 Cys residues and they do not contain aromatic amino acid residues or His. All the Cys residues are involved in the binding of seven divalent (Zn(II), Cd(II)) and up to 12 monovalent metal ions such as Cu(I) through two distinct metal-thiolate clusters located in two independent protein domains termed the α and β domains (Kagi and Kojima, 1987, Vasak and Hasler, 2000, Romero-Isart and Vasak, 2002). The suggested functions of mammalian MTs include the maintenance of homeostasis and transport of physiologically essential metals (zinc, copper), detoxification of toxic metals (cadmium, mercury), protection against oxidative stress, regulation of cell proliferation and apoptosis, and the regulation of intracellular redox balance (Maret, 2000, Palmiter, 1998, Miles et al., 2000, Hidalgo et al., 2001, Coyle et al., 2002, Kang, 2006). To maintain copper ion homeostasis under conditions of copper excess or deficiency (Rae et al., 1999, Ogra et al., 2006), many eukaryotic organisms express MTs that can buffer the cellular metal fluctuations. Although MTs cannot directly export excess copper from the cell, it has been suggested that they can store copper, and may constitute a reservoir under conditions of temporary copper deficiency (Ogra et al., 2006). The importance of MTs in detoxification of heavy metals is demonstrated in studies of the exposure of MT-1 and MT-2 (MT-1/2) knockout mice to heavy metals, which leads to metal toxicity, while MT-1/2 overexpressing mice are relatively well protected from heavy metal toxicity (Coyle et al., 2002). MTs have important roles in copper homeostasis, evidenced by the crossing of a mouse model of Menkes' disease with MT-1/2 knockout mice, which results in embryonic lethality (Kelly and Palmiter, 1996).

In mammals MT-1 and MT-2 show ubiquitous expression regulated at the transcriptional level (Lichtlen and Schaffner, 2001). Their biosynthesis is induced by a variety of stress conditions and compounds such as metals, glucocorticoids, cytokines, and reactive oxygen species (Miles et al., 2000, Davis and Cousins, 2000). Expression of MT-1 and MT-2 is also enhanced under Cu-deficient conditions in order to maintain the activities of intracellular cuproenzymes such as CCO and SOD1 (Ogra et al., 2006). MT-3 and MT-4 are relatively insensitive to these inducers. Expression of MT-3 is largely restricted to the central nervous system, where it represents the major component of the intracellular Zn(II) pool in zinc-enriched neurons (Masters et al., 1994). Expression of MT-3 at lower levels occurs in the pancreas, kidneys, reproductive tissues and maternal deciduum. Expression of the last-identified mammalian metallothionein isoform, MT-4, is restricted in mice to cornified, stratified, squamous epithelium, a tissue providing a protective surface on the skin, footpad, tail, tongue, the upper part of the alimentary tract, and the vagina (Quaife et al., 1994). Expression of MT-4, together with proteins of the entire MT gene locus, is developmentally regulated in maternal deciduum in mice (Liang et al., 1996). Expression of MT-4 is also regulated by the transcription factor Whn together with other proteins involved in the metabolism of keratin (Schlake and Boehm, 2001).

It has been demonstrated that MT-1/2 expression in astrocytes is significantly upregulated in regions of Aβ plaque pathology in the brains of patients with Alzheimer's disease (Richarz and Bratter, 2002, Zambenedetti et al., 1998), as well as in the brains of a mouse model of Alzheimer's disease (Carrasco et al., 2006). MT-1/2 proteins are characterized as highly neuroprotective proteins essential for brain repair and their expression is dramatically increased in response to various central nervous system injuries (Penkowa et al., 1999, Chung et al., 2003, Chung et al., 2008). In contrast to MT-1/2, the brain-specific isoform MT-3 is downregulated in Alzheimer's disease brain (Sogawa et al., 2001).

1.6. Cu(I)-binding affinities of cytoplasmic copper proteins

A key property of the copper-binding proteins is their metal affinity, defined by the dissociation constant (K_d) of the corresponding metal-protein complex (Equations 1 and 2). Reliable evaluation of metal-binding affinities is important for a more detailed understanding of the cellular metal selection and speciation mechanisms. However, estimation of these metal-binding constants for Cu(I) is associated with various problems, which is reflected by the appearance of very disparate values for the affinities of the same proteins in the literature (Zimmermann et al., 2009a, Xiao and Wedd, 2010).

$$Cu^{+} + P \xleftrightarrow{K_D} Cu^{+}P \qquad (1)$$

$$K_D = \frac{\left[Cu^{+}\right] \times \left[P\right]}{\left[Cu^{+}P\right]} \qquad (2)$$

The predominant copper oxidation state under reducing intracellular conditions is Cu(I). However, maintaining the redox state of Cu(I) is not an easy task. In addition to the instability of Cu(I) ions in mild oxidative conditions, there are several other pitfalls and complicating factors, all of which must be avoided or kept under control: a) Lack of effective competition in equilibrium experiments. b) Lack of effective control of pH and its influence upon the affinities of ligand probes. (c) The fact that Cu(I) is prone to disproportionation ($2Cu(I) \leftrightarrow Cu(II) + Cu(0)$) in aqueous solution. Ligands that form stable Cu(I) complexes (e.g., acetonitrile, DTT, TCEP) can prevent this complicating reaction that introduces Cu(II), however, the equilibria involving complex formation of Cu(I) with these stabilizing ligands need to be considered in thermodynamic analysis (Wilcox, 2008, Miras et al., 2008). (d) Lack of effective control of redox milieu and oxidation of Cys residues in metal-binding sites. (e) The presence of ternary complexes in the equilibrium mixture. (f) Incorrect interpretation and processing of the experimental data (Xiao and Wedd, 2010).

1.6.1. Methods used to determine Cu(I)-binding affinities

The metal-binding affinities of almost all Cu(I)-binding proteins for copper ions are too high for direct determination by titration, and therefore indirect methods have to be used. A synthetic metal-binding ligand can provide a reliable probe for estimation of metal-binding affinities if effective competition can be induced between the protein and the ligand for the metal ion and if the essential concentrations can be quantified (Zimmermann et al., 2009a). In principle, there are two possibilities for quantification; first, direct assessment of the Cu(I)-protein complex using ESI-MS, Cd^{2+}-induced UV absorbance, NMR or some other method that is sensitive to changes in the protein molecule upon Cu(I) binding and second, determination of the amount of Cu(I)-ligand complex in the solution by using Cu(I)-binding colored or fluorescent dyes as competing ligands.

a) Spectrophotometric methods

Two chromophoric Cu(I)-specific ligands, bicinchoninic acid (BCA) and bathocuproine disulfonate (BCS), have commonly been used in studies of Cu(I)-binding proteins (Xiao et al., 2004, Yatsunyk and Rosenzweig, 2007, Hussain et al., 2008, Zimmermann et al., 2009a, Chong et al., 2009, Miras et al., 2008, Koay et al., 2005, Zhang et al., 2006, Zhou et al., 2008, Xiao et al., 2008). Both of these ligands form colored 1:2 complexes with Cu(I) ions. Transfer of Cu(I) to protein (P) is determined from changes in absorbance at 483 nm for L=BCS and at 562 nm for L=BCA, corresponding to concentrations of $[Cu(I)(BCA)_2]^{3-}$ and $[Cu(I)(BCS)_2]^{3-}$, respectively (Xiao et al., 2004, Xiao et al., 2008). The difference in the formation constants of the two complexes ($\beta_2(BCA)=K_1*K_2=10^{17.2}$ M^{-2} and $\beta_2(BCS)=K_1*K_2=10^{19.8}$ M^{-2}) allows reliable estimation of Cu(I)-binding affinities within a wide K_d range from 10^{-11} (10^{-12}) to 10^{-19} M (Xiao et al., 2008, Xiao et al., 2004, Yatsunyk and Rosenzweig, 2007, Zhou et al., 2008, Zimmermann et al., 2009a, Xiao and Wedd, 2010). Application of BCA and BCS is connected to a complicated two-step binding scheme for Cu(I) and the possibility of formation of ternary complexes, which complicates the determination of Cu(I)-binding affinities of proteins.

Fluorescence spectroscopy has been used for determination of the Cu(I) affinity of *E. hirae* CopZ. Cu(I) was titrated to Cd(II)-bound protein and the decrease in fluorescence of the Cd(II)-protein complex was followed (Urvoas et al., 2004).

b) Mass spectrometric methods

Electrospray ionization mass spectrometry (ESI-MS) (Fenn, 2003) offers an opportunity to monitor protein-ligand interactions directly, i.e., by transferring the corresponding complexes into the gas phase such that they can be detected in a mass spectrum (Ganem et al., 1991, Katta and Chait, 1991, Marco and Bombi, 2006). The advantage of the method is that multiple co-existing forms and the metal-binding stoichiometry of the complexes can be determined from the detected mass spectrum (Palumaa et al., 2004, Benesch et al., 2007, Loo, 2000, Smith et al., 2006), which can be used for calculation of metal-binding affinities (Jecklin et al., 2008, Daniel et al., 2002, Gabelica et al., 2003). Because of the high sensitivity of modern mass spectrometers the studies require small amounts of sample (Pan et al., 2009). Another important advantage of the ESI-MS technique is that dithiothreitol (DTT) can be used as a competing Cu(I) ligand. DTT is an excellent ligand for the determination of Cu(I)-binding affinities using the ESI-MS technique, since, being a nonionic compound it is applicable in ESI-MS experiments without affecting the intensity of spectra (Palumaa et al., 2004). Moreover, the Cu(I)-binding constant of

DTT is suitable for these studies (7.9 x 10^{-12} M (Krezel et al., 2001)) and DTT can, at supramillimolar concentrations, extract metals from copper proteins (Palumaa et al., 2004). It is also relevant that DDT forms a 1:1 complex with the Cu(I) ion, which simplifies the calculations and minimizes the risk of ternary complex formation.

c) Spectroscopic methods

In a few cases NMR spectroscopy has also been used for the determination of Cu(I)-binding affinities (Banci et al., 2007b, Abriata et al., 2008). In these studies DTT has been used in the role of reducing agent and competing ligand for Cu(I). Cu(I)-binding affinity has been estimated through changes in heteronuclear single quantum correlation (HSQC) spectra recorded after addition of increasing concentrations of DTT to Cu(I)-loaded protein (Abriata et al., 2008) or after addition of Cu(I) to apo-protein (Banci et al., 2007b). An attempt has been made to use circular dichroism (CD) for Cu(I) affinity determination by direct titration of the protein with copper ions (DeSilva et al., 2005).

d) Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is also considered as a suitable method to measure binding equilibria for the d^{10} closed-shell spectroscopically-challenged Cu(I) ion (Wilcox, 2008). ITC detects the change in heat content upon titration of metal ion into apo-protein solution and this technique can be applied to measure metal-protein affinities (Velazquez-Campoy et al., 2004, Wilcox, 2008). As with any method measuring a bulk property, especially one as ubiquitous as heat, experiments need to be carefully designed, necessary control measurements need to be made, all contributions to the experimental signal need to be considered, appropriate models need to be used in fitting the data, and careful analysis to account for any coupled or competing reactions need to be included in analysis of the data (Wilcox, 2008). The observed change in heat can include contributions from associated equilibria and so careful assessments of the influence of redox, precipitation and hydrolysis equilibria, plus the metal- and proton-binding capacities of the buffer must be included. Although ligands that form stable Cu(I) complexes can prevent Cu(I) disproportionation, equilibria involving these Cu(I)-stabilizing ligands need to be considered in thermodynamic analysis. These aspects can lead to significant errors in estimates of K_d (Wilcox, 2008, Xiao and Wedd, 2010).

1.6.2. Metal-binding affinities of individual Cu(I)-binding proteins

The dissociation constant values for the same Cu(I) target determined by different methods vary by several orders of magnitude depending on the experimental conditions and method used (Table 2). The most representative example as regards the complications in affinity measurements is the human copper chaperone HAH1 (Atox1), for which the dissociation constant estimates vary by thirteen orders of magnitude (Wernimont et al., 2004, Hussain et al., 2008) (Table 2). Some of the affinity estimates contradict the generally accepted route of copper trafficking. For instance, the estimate for the Cu(I)-binding affinity of the yeast Cu(I) importer Ctr1 (K_d = 3.1 x 10^{-19} M) (Xiao et al., 2004) is higher than estimates for the yeast Cu(I) chaperone Atx1 (K_d = 2.1 x 10^{-18} M, and K_d= 4.2 x 10^{-17} M) (Xiao et al., 2011, Miras et al., 2008). It is accepted that Atx1 receives Cu(I) from Ctr1 (Xiao and Wedd, 2002, Bertini and Cavallaro, 2008). However, when Ctr1 has a higher affinity for Cu(I) than Atx1 does, the transfer cannot occur. The K_d values acquired for ATPase metal-binding domains (10^{-6} M, 10^{-7} M, 10^{-11} M, 10^{-18} M) (Wernimont et al., 2004, Jensen et al., 1999, Yatsunyk and Rosenzweig, 2007, DeSilva et al., 2005, Xiao et al., 2011) differ by many orders of magnitude. Affinity values for the bacterial copper chaperone CopZ also differ significantly (1.2 x 10^{-7} and 1.7 x 10^{-22} M) (Kihlken et al., 2002, Zhou et al., 2008). Considering the huge variation, it can be concluded that reliable estimates of K_d values for different Cu(I)-binding proteins should be assessed by a single method, using similar experimental conditions.

Table 2. Dissociation constants of intracellular Cu(I)-binding proteins and their metal-binding domains (MBDs) from the literature.

Protein	K_d, M	Method, conditions	Reference
$Cox17_{2S\text{-}S}$ (porcine)	6.4 x 10^{-15}	ESI-MS; 20 mM ammonium acetate, pH 7.6. 1.4 μM Cox17, 0.15–0.35 mM DTT as SH agent; 0–10 mM DTT, 0–16 mM GSH and 1.8 μM Cox17 were used for Cu(I) affinity determination.	(Palumaa et al., 2004)
$Cox17_{0S\text{-}S}$ (porcine)	13 x 10^{-15}		
Cox17 (yeast)	1.62 x 10^{-7}	ITC; 100 mM MES, 100 mM NaCl, pH 6.5. Stirred at 400 rpm, 25 °C, anaerobic. 5–25 μM protein and 200–600 μM Cu(I)Cl solution.	(Abajian et al., 2004)
Sco1 (human)	~10^{-17}	NMR spectroscopy, HSQC; apoSco1 was titrated with $Cu(I)Cox17_{2S\text{-}S}$ or $[Cu(I)(CH_3CN)_4]PF_6$, 1 mM DTT. Coy chamber under nitrogen atmosphere.	Banci et al. 2007

Sco1 (*T. hermophilus*)	~10^{-10}	NMR spectroscopy, HSQC; $[Cu(I)(CH_3CN)_4]PF_6$, 100 mM potassium phosphate, pH 7, increasing concentrations of DTT.	(Abriata et al., 2008)
PCu_AC (*T. thermophilus*)	2.2 x 10^{-13}	NMR spectroscopy, HSQC; 50 mM phosphate buffer, pH 7.2, 1 mM ascorbate, $[Cu(I)(CH_3CN)_4]PF_6$, increasing concentrations of DTT. K_d (DTT) = 6.3 x 10^{-12} M.	(Abriata et al., 2008)
Cu_A (Cox2) (*T. thermophilus*)	~10^{-15}	NMR spectroscopy, HSQC; $[Cu(I)(CH_3CN)_4]PF_6$, 50 mM phosphate buffer, pH 7.2, 1 mM ascorbate, anaerobic conditions, increasing concentrations of DTT.	(Abriata et al., 2008)
SOD1 (yeast)	6 x 10^{-15}	BCS; 1.8 μM apoSOD1, 50 mM mM potassium phosphate, pH 7.8, 20 μM $ZnSO_4$, 1 mM GSH. 10 μM Cu(I)CCS or 10 μM $Cu(I)(CH_3CN)_4PF_6$ and 0 or 200 μM BCS. K_d (BCS) ~ 10^{-20} M.	(Rae et al. 1999)
Atox1 (human)	4.08 x 10^{-6}	ITC; 100 mM MES, 100 mM NaCl, pH 6.5. Stirred at 300 rpm, 22 °C, anaerobic. 25 μM Atox1 0.5 mM Cu(I); 4.2 μM WD16, 0.6 mM Cu(I); 8.4 μM WD12, 0.25 mM Cu(I); 8 μM WD34, 0.5 mM Cu(I); 8 μM WD56, 0.4 mM Cu(I).	(Wernimont et al., 2004)
ATP7B MBD1-6 (human)	5.10 x 10^{-6}		
MBD1-2	4.83 x 10^{-6}		
MBD3-4	2.14 x 10^{-7}		
MBD5-6	8.84 x 10^{-7}		
ATP7B MBD1 (human)	3.85 x 10^{-11}	BCA; Spectrophotometric; 0.1 molar eq of reduced apoproteins (1–4 μl, 0.3–0.6 mM) were titrated into 0.5–0.7 ml of 10 μM Cu(I) solution in the presence of 0.5–1 mM BCA. Degassed 50 mM HEPES, 200 mM NaCl, pH 7.5, 0.2 mM ascorbate. Incubation time 10 min after each addition of protein. K_2- second Atox molecules binds to CuAtox. Their BCA β_2 = 4.6 x 10^{14} M^{-2}.	(Yatsunyk and Rosenzweig, 2007)
MBD2	2.86 x 10^{-11}		
MBD3	1.59 x 10^{-11}		
MBD4	4.00 x 10^{-11}		
MBD5	4.55 x 10^{-11}		
MBD6	1.72 x 10^{-11}		
Atox1	2.86 x 10^{-11} 2.94 x 10^{-7}		
ATP7A hisMBD1 (human)	38.8 x 10^{-6}	Dialysis, under anaerobic conditions; Cu(I) solution: 2–212 μM $CuCl_2$ in 10 mM ascorbic acid, 50 mM Tris-HCl, 1 M NaCl, pH 7.4. Buffer: 50 mM Tris-HCl, 1 M NaCl, pH 7.4.	Jensen et al. 1999
hisMBD1-2	65.2 x 10^{-6}		
hisMBD1-6	19.2 x 10^{-6}		
ATP7A MBD1 (human)	1.8 x 10^{-6}	CD; at 200 nm. 0.08 mM MNK D1 in 20 mM sodium acetate, 0.1 mM DTT, pH 6.5. (Cu-sulfate)	DeSilva et al. 2005
ATP7A MBD1 (human)	45.5 x 10^{-6}	Dialysis, under anaerobic conditions; 40 μM MBD1, 200 μM Cu(I) solution ($CuCl_2$ in 10 mM ascorbic acid, 50 mM Tris-HCl, 1 M NaCl, pH 7.4). Buffer: 50 mM Tris-HCl, 1 M NaCl, pH 7.4.	Jensen et al. 1999 (FEBS)

Ctr1 (yeast)	3.1 x 10^{-19}	BCS; 20 mM Tris/Mes pH 8, $[Cu(I)(MeCN)_4]ClO_4$, anaerobic conditions, 1 mM GSH as reducing agent. Final concentrations 5 μM Ctr1, 15μM Atx1 and Ccc2, 30 μM Cu(I), 1–2 mM BCS.	Xiao et al. 2004
Atx1 (yeast)	5.7 x 10^{-19}		
Ccc2 (yeast)	1.5 x 10^{-19}		
Atx1 (yeast)	4.17 x 10^{-17}	BCS (485 nm); Cu(I) as Cu_2SO_3: Cu(II) + Na_2SO_3. 50 mM Mes-NaOH pH 6, 1 mM Na_2SO_3, 400 mM NaCl, $CuSO_4$.	Miras et al. 2008
Atox1 (human)	6.5 x 10^{-19}	BCA (565 nm); CuAtox1 was titrated with increasing amounts of BCA. 3 μM Atox1, 20 mM Tris, 150 mM NaCl, 0.5 mM DTT, pH 7.5, 20 °C. Log $K_{stability}$ (BCA) ≈14.	(Hussain et al., 2008)
Atx1 (yeast)	2.1 x 10^{-18}	BCS; $[Cu(I)(CH_3CN)_4]ClO_4$. Anaerobic glove-box, 1 mM GSH. 36 μM Cu(I), 500, 300, 200 μM BCS, 10–60 μM Atx1, 15–60 μM WND5-6 (titration with protein). KPi buffer, 25 mM, 100 mM NaCl, pH 7.0.	(Xiao et al., 2011)
WND MBD5-6 (human)	4.0 x 10^{-18}		
CopZ (*B. subtilis*)	1.2 x 10^{-7}	UV-vis spectroscopy; Abs at 265 nm; CuCl titrations were done under anaerobic conditions, incubated 5 min. 50 mM Mops, pH 7.5, 33 μM CopZ, 0–1.72 Cu(I) ions per protein, 25 °C.	(Kihlken et al., 2002)
CopZ (*B. subtilis*)	1.75 x 10^{-22}	BCS, BCA; anaerobic conditions, CuCl in 1M NaCl and 100 or 10 mM HCl. 100 mM Mops, 100 mM NaCl, pH 7.5. BCS (483 nm); β_2(CopZ) = 1.1 x 10^{22} β_2 (BCS) = 6.3 x 10^{19} M^{-2}. BCA (562 nm); β_2(CopZ) = 2.4 x 10^{20} β_2 (BCA) = 4.6 x 10^{14} M^{-2}.	(Zhou et al., 2008)
CopZ (*E. hirae*)	≤10^{-12}	Fluorescence spectroscopy; Cu(I)Cl (in 0.1 M HCl / 1M NaCl or in complex with acetonitrile, under argon) was added to the CdCopZ complex. 20 mM Mops, 150 mM NaCl, pH 7.2, 22 °C.	(Urvoas et al., 2004)
CopC (*P. syringae*)	≥10^{-13}	BCS; 15 μM Cu(I), 45 μM BCS, increasing amount of apoCopC (0–750 μM), 20 mM Mes, 100 mM NaCl, pH 6, anaerobic conditions.	Koay et al. 2005
CopC (*P. syringae*)	≥10^{-13}	BCS (483); anaerobic conditions, 15 μM Cu(I), 45 μM BCS, 20 mM Mes, 100 mM NaCl, pH 6. Increasing concentrations of apoCopC. β_2 = $10^{19.8}$ M^{-2}.	(Zhang et al., 2006)
CopK (*C. metallidurans*)	2 × 10^{-11}	BCA (358); $[Cu(I)(CH_3CN)_4]ClO_4$ under anaerobic conditions, 500 μM ascorbate. 20 mM Tris/Mes, 100 mM NaCl, pH 8. β_2(BCA) $10^{17.2}$ M^{-2}.	(Chong et al., 2009)

HMA2n (1-79) (P_{1B} type ATPase) (*A. thaliana*)	1.9 x10^{-17}	BCA (562 nm), BCS (483 nm); anaerobic, $[Cu(I)(MeCN)_4]^+$. 50 mM Mops, 100 mM NaCl, pH 7.3. 30 μM Cu(I), 500 μM BCS. β_2(BCA) $10^{17.2}$ M^{-2}, β_2(BCS) $10^{19.8}$ M^{-2}.	(Zimmermann et al., 2009a, Zimmermann et al., 2009b)
HMA4n (1-96)	2.9 x 10^{-17}		
HMA7n (56-127)	6.5 x 10^{-19}		
CueR (*E. coli*)	10^{-21}	ICP-AES; $[Cu(CH_3CN)_4]PF_6$ The Cu(I) solution was kept in N_2 and 1 mM DTT was added to the solution. Incubation buffer, pH 8; 20, 5 and 1 mM CN^-.	(Changela et al., 2003)
CueO T4 (*E. coli*)	1.3 x 10^{-13}	BCA; $[Cu(I)(CH_3CN)_4]^+$, 50 mM Bis-Tris-HCl, pH 7. β_2(BCA) $10^{17.2}$ M^{-2}	(Djoko et al., 2010)
DTT	5 x 10^{-16} (pH 7.3)	BCS; $[Cu(I)(CH_3CN)_4]ClO_4$. Anaerobic glove-box. 50 mM Mops, 4 mM Na-dithionite, pH 7.3 or pH 6.8, 21 μM Cu(I), 50 μM BCS. 50 μM BCS, 21 μM Cu(I).	(Xiao et al., 2011)
DTT	2.5 x 10^{-15} (pH 6.8)		
DTT	7.9 x 10^{-12}	Potentiometry; 0.1 M KNO_3, pH 7.4, 25 °C.	(Krezel et al., 2001)
BCS	$\beta_2 = 10^{19.8}$	BCS; 20 mM Tris/Mes pH 8, $[Cu(I)(MeCN)_4]ClO_4$, anaerobic conditions, 1 mM GSH as reducing agent. 1–2 mM BCS.	(Xiao et al., 2004)
BCA	$\beta_2 = 10^{17.2}$	BCS; 20 mM KPi buffer, 100 mM NaCl, pH 7, 1 mM Na-dithionite, 30% DMSO, anaerobic.	(Xiao et al., 2008)
BCA	$\beta_2 = 10^{14.7}$	ITC; anaerobic, 50 mM Hepes, 200 mM NaCl, pH 7.5; 75-80 μM Cu(I), 0.2 mM ascorbate. 400 rpm.	(Yatsunyk and Rosenzweig, 2007)

2. AIMS OF THE STUDY

The main aim of the current study was to create a systematic overview of cellular copper trafficking and distribution. For this purpose the specific aims were:

1. To adopt ESI-MS methodology for determination of Cu(I)-binding constants for different cellular Cu(I)-binding proteins.
2. To determine reliable Cu(I) affinity constants for representative members of the cellular Cu(I) proteome.
3. To adopt ESI-MS methodology to measure Zn(II)-binding constants of cellular Zn-binding proteins and demonstrate its broader applicability for determination of Zn(II)-binding constants of cellular Zn-proteins.

3. MATERIALS AND METHODS

PUBLICATION I

- ✓ Protein expression and purification (MT-2)
- ✓ Reconstitution of proteins with Cu(I) ions
- ✓ Determination of metal-binding equilibria in the presence of DTT
- ✓ ESI-MS measurements
- ✓ Determination of dissociation constants of Cu(I)-ligand complexes
- ✓ Extraction of Cu(I) from Cu,Zn-SOD1
- ✓ Calculation of dissociation constants of Cu-protein complexes

PUBLICATION II

- ✓ ESI-MS studies of Cu(I) binding to MT-3
- ✓ Determination of the Cu(I) dissociation constant of MT-3

PUBLICATION III

- ✓ Elaboration of ESI-MS methodology for determination of Zn(II)-binding constants
- ✓ Determination of Zn(II)-binding affinity of Cox17 redox forms

4. RESULTS AND DISCUSSION

To achieve better understanding of intracellular copper trafficking and distribution, the Cu(I)-binding affinities of cellular copper proteins were determined. In order to obtain information about all three main routes of copper trafficking inside the cell, the key copper proteins involved in these routes were selected: (i) the Cu_A domain of CCO as the copper target and copper chaperones for CCO – Cox17, Sco1 and Sco2; (ii) the antioxidant enzyme Cu,Zn-SOD1 (SOD1) and the copper chaperone for SOD1 – CCS; and (iii) N-terminal domains of ATP7A (Menkes' disease protein) and the chaperone HAH1. Furthermore, the Cu(I)-binding affinities of two important intracellular metal-binding proteins, metallothionein-2 and -3 (MT-2 and MT-3) as well as those of the low-molecular-mass thiol ligands glutathione (GSH) and diethyldithiocarbamate (DETC) were determined.

An ESI-MS-based approach was used for affinity studies. This method relies on continuous monitoring of the metalated to non-metalated protein ratio in the presence of a competing Cu(I)-binding ligand. As a rule, dithiothreitol (DTT) was used as the competing ligand, but in the case of proteins with extra high affinity, DETC was used. For determination of dissociation constants, first, the concentrations of free metal ions in the presence of increasing concentrations of competing ligand were calculated, and second, the fractional content of metalated protein was determined for each DTT concentration from the ESI-MS spectra and correlated with the concentration of free copper ions in solution. The obtained curves were nonlinearly fitted using a hyperbolic equation corresponding to a simple 1:1 binding equilibrium, or a more complicated equation if necessary, and the dissociation constants were attained. The equilibrium between the apo- and metalated protein form was reached within 2 min of incubation, which is the minimal time for a measurement in ESI-MS.

The ESI-MS-based method has several advantages: (i) universal applicability to a large set of copper proteins with different metal-binding stoichiometries, affinities and binding schemes; (ii) the experiments are usually performed in the presence of DTT, which creates reducing conditions mimicking the cellular redox environment; (iii) DTT forms a stable complex with Cu(I) ions, preventing their oxidation or disproportionation; (iv) the apparent Cu(I)-binding affinity of DTT, used as reference to obtain all of the other apparent Cu(I)-binding constants, is known (Krezel et al., 2001) and is such that DTT at millimolar concentrations can effectively compete with most of the Cu(I)-binding proteins present at micromolar concentrations; (v) the metal-binding stoichiometry of the various copper-binding molecules simultaneously present in solution can be determined. ESI-MS-based

approach can also be applied to other metal ions, as demonstrated in the case of Zn binding to Cox17 (Publication III, Figs. 7 and 8).

The apparent Cu(I) and Zn(II) dissociation constants for DTT are known (Krezel et al., 2001), but no reliable Cu(I)-binding constants have been reported for GSH and DETC. Apparent Cu(I)-binding constants for GSH and DETC were determined through parallel experiments with GSH and DTT, and with DETC and DTT. The known Cu(I)-affinity constant for DTT was used to obtain the constants for GSH and DETC. Parallel experiments with Cu_IHAH1 and Cu_ICox17 in competition with GSH or DTT revealed that GSH and DTT have similar affinities for Cu(I) (Publication I, Fig. 1a,b), Table 3. As GSH is an abundant low-molecular-weight thiol present at millimolar concentrations in cytosol of eukaryotic cells (Ostergaard et al., 2004), it has an important function in determining the intracellular redox environment. Millimolar concentrations of DTT can thus correctly imitate the Cu(I)-binding capacity of GSH and mimic the cellular redox milieu. The Cu(I)-binding affinity of DETC was estimated through experiments with Cu_ISco1, where DTT or DETC was added to the metalated Sco1. The Cu(I)-binding affinity of DETC was approximately 400 times higher than the affinity of DTT (Publication I, Fig. 1c), (Table 3).

The apparent metal-binding constants of all the selected proteins, except SOD1, MT-2 and MT-3, were determined using DTT as a competing metal-binding ligand. The Cu(I)-binding constants of SOD1 and the MTs were determined through competition with DETC. The apparent dissociation constants for intracellular Cu(I)-proteins fell into the femtomolar (fM) range and are presented in Table 3 (Publication I, Table1, Supplementary Fig. S3-13; Publication II, Fig. 2A).

The apparent Cu(I)-binding constants presented in Table 3 indicate that the copper chaperones HAH1 (16.8 fM) and Cox17 (17.4 fM) have similar affinities for Cu(I). The abundant low-molecular-mass cellular thiol ligand GSH has a dissociation constant of 9.1 pM, and at millimolar concentrations in the cell it has comparable Cu(I)-binding capacity, constituting an exchangeable cellular copper pool. The Cox17 partners in the mitochondrial intermembrane space are the proteins Sco1 and Sco2, and their Cu(I)-binding affinities (3.1 and 3.7 fM, respectively) are about five times higher than that of Cox17. The affinity of the Cu_A site of CCO (0.73 fM) is more than four times higher than those of the chaperones Sco1 and Sco2, supporting the accepted routes of copper trafficking inside the cell and showing the Cu(I) trafficking from chaperones to co-chaperones towards the enzyme The copper chaperone CCS has the highest affinity for Cu(I) among cytoplasmic Cu(I) chaperones (2.4 fM), which is seven times higher than the affinities of HAH1 and Cox17. CCS is the Cu(I) chaperone for SOD1. As the affinity of SOD1 for copper (0.23 fM) is more than ten times higher, this direction of Cu(I) trafficking is thermodynamically supported. HAH1 delivers Cu(I) to ATP7A metal-binding

domains (MBDs). The five separate MBDs analyzed revealed different affinities for Cu(I). Domains 1 (2.9 fM), 2 (4.9 fM) and 6 (2.6 fM) had Cu(I)-binding affinities three to seven times higher than that of the chaperone HAH1 (16.8 fM). Domain 5 (13 fM) had only a slightly higher affinity and domain 3 (104 fM) had a lower affinity than HAH1 (Table3) (Publication I).

Table 3. Apparent dissociation constants (±SD) for Cu(I)-binding proteins and low-molecular-mass ligands (Publications I and II).

Protein/ligand	K_{Cu} (x 10^{-15} M)	R^2†
HAH1	16.8 ± 4.8	0.89
Cox17 (2S-S)	17.4 ± 2.3	0.96
ATP7A MBD1	2.5 ± 0.5	0.88
ATP7A MBD2	4.9 ± 1.4	0.73
ATP7A MBD3	104 ± 44	0.82
ATP7A MBD5	13.0 ± 2.9	0.93
ATP7A MBD6	2.6 ± 0.6	0.84
CCS	2.4 ± 0.2	0.96
Sco1	3.1 ± 0.7	0.94
Sco2	3.7 ± 0.5	0.95
Cu_A site of Cox2 ‡	0.73 ± 0.07	0.86
Cu site of SOD1 £	0.23 ± 0.02	0.95
MT-2	0.41 ± 0.04	0.97
MT-3 §	0.47 ± 0.01	
DTT ¶	7940	
GSH #	9130	0.84
DETC ♣	13.8 ± 0.2	0.81

† Describes the quality of the fit.
‡ Protein from *T. thermophilus*. All the other proteins are human proteins.
£ Calculated from demetalation experiments of Cu,Zn-SOD1 with DETC.
§ From Publication II, all the other constants are from Publication I.
¶ Taken from reference (Krezel et al., 2001).
# Calculated from the comparison of the Cu(I) affinity of GSH with that of DTT.
♣ Calculated from the comparison of the Cu(I) affinity of DETC with that of DTT.

The N-terminal domains of Cu-ATPases are suggested to be the sensors of intracellular copper concentrations and to regulate the ATPase activity or localization accordingly (Voskoboinik et al., 2001, Tsivkovskii et al., 2001, Banci et al., 2010, DiDonato et al., 2000, Petris et al., 2002). It has been suggested that in copper deficiency N-terminal MBDs can associate with ATP-binding domain and reduce the catalytic activity of ATPase (Tsivkovskii et al., 2001) or can be involved in capturing copper ions and supplying them to the ATPase (Voskoboinik et al., 2001). When intracellular copper concentrations are elevated the relocation

of Cu-ATPases to the plasma membrane will be initiated (Mercer et al., 2003, Veldhuis et al., 2009, Hung et al., 1997, Petris et al., 1996, Monty et al., 2005). It has been shown that only MBDs 5 and/or 6 but not 1 to 4 are important for relocation machinery (Strausak et al., 1999, DiDonato et al., 2000, Mercer et al., 2003, Cater et al., 2004, Guo et al., 2005). Our results indicate a special role for domain 5 in the relocation machinery, as this domain had one of the lowest affinities for Cu(I) among N-terminal MBDs, but higher affinity than the chaperone HAH1 does, suggesting that when the intracellular concentration of Cu(I) is so high that even domain 5 gets Cu(I), then there is a need to relocate ATPase to the vesicular membrane and eventually to the plasma membrane for extensive copper export. The higher affinity of domain 6 can be explained by the essential role of this domain in copper insertion into the *trans*-Golgi network. It has been shown that not all six MBDs are necessary for correct Cu(I) translocation activity of ATPase to the *trans*-Golgi network, although domain 6 can play an important part in correct functioning of the ATPase (Forbes et al., 1999, Cater et al., 2004). To bring it together, the cellular copper chaperones and their target proteins have Cu(I)-binding affinities that thermodynamically drive copper ions to their destinations – to the enzymes that need copper for their function (Fig. 12). Therefore, a distinct hierarchy exists among all the Cu(I)-binding proteins in the cell, from chaperones to intermediate copper chaperone-proteins and finally to enzymes. However, the hierarchy is not the only reason for targeted Cu(I) delivery. Other important factors in intracellular copper delivery are specific protein–protein interactions that overcome the thermodynamic hierarchy and eliminate non-targeted delivery.

Interestingly, very high Cu(I)-binding affinities were observed for MTs (Table 3). MT-2A revealed a Cu(I) dissociation constant of 0.41 fM (Publication I, Fig. S14b), and MT-3 had only a slightly lower affinity, (K_d = 0.47 fM) (Publication II, Fig. 2A). However, differences were found in metal-binding properties and affinities of copper-thiolate clusters of MT-2A and MT-3. The major Cu(I)-bound form of MT-2A was Cu_{10}MT-2 (Publication I, Fig. S14a), and MT-3 had a mixture of 10–12 Cu(I)-bound ions, i.e. Cu_{10}MT-3, Cu_{11}MT-3 and Cu_{12}MT-3 (Publication II, Fig. 2B). The Cu(I)-binding affinities of the different clusters were also different. Cu_{10}MT-2A revealed dissociation of 4-metal clusters and the appearance of Cu_6MT-2A forms in the presence of 1.5 mM DETC (Publication I, Fig. S14a). The 6-metal cluster form of MT-3 dissociated in the presence of 0.5 mM DETC, revealing the Cu_6MT-3 form (Publication II, Fig. 2B). This suggests that the 4-metal cluster form in the Cu_{10}MT-2A α-domain has higher Cu(I)-binding affinity than the 6-metal cluster form in the Cu_{12}MT-3 α-domain and therefore contributes to the overall higher affinity of MT-2A for Cu(I) ions. Both 6-metal clusters in Cu_6MT-2A and Cu_6MT-3 in β-domains dissociated in the presence of 3 mM DETC (Publication I, Fig. S14a; Publication II, Fig. 2B) and share similar metal-binding properties. When apoMT-2A was added to the copper proteins discussed above, it

extracted metals from all the proteins (Publication I, Fig. 2A) except the copper enzymes. ApoMT-2A is not capable of taking copper away from Cu,Zn-SOD1 even at a twofold molar excess of apoMT-2A over a period of 3 hours. The reason for that lies probably in the inaccessibility of the metal sites of SOD1 to MT. Although apoMT-2A was able to extract copper from the Cu_A site of prokaryotic Cox2 (Publication I, Fig. 2B), it was not able to inactivate the membrane-bound CCO (Publication I, Fig. S17), probably for kinetic reasons, as found for Cu,Zn-SOD1.

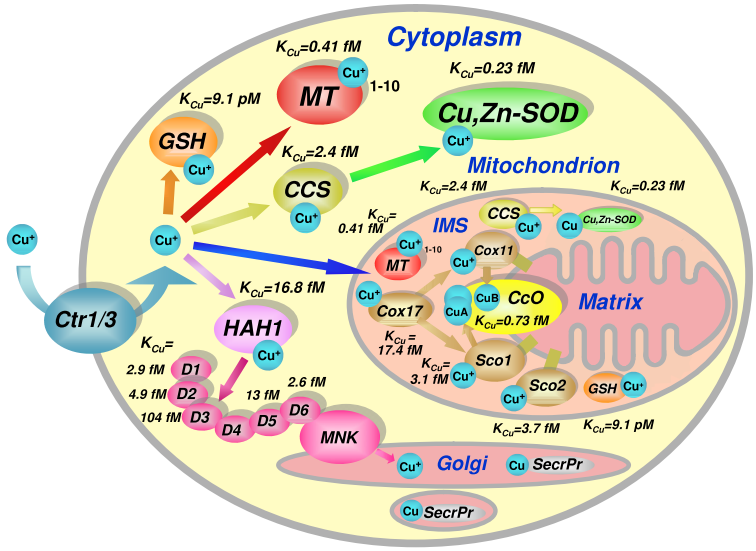

Figure 12. Copper trafficking pathways in a eukaryotic cell. Blue spheres – Cu ions; Ctr1/3 – copper influx transporters; CCS – copper chaperone for SOD1; HAH1 – copper chaperone for Menkes' protein; D1–D6 – Cu(I) binding domains of Menkes' protein; MT – metallothionein; GSH – glutathione; Cox11, Cox17, Sco1, Sco2 – copper chaperones and co-chaperones for cytochrome c oxidase; CCO - cytochrome c oxidase; SecPr – secreted copper proteins, such as ceruloplasmin, lysyl oxidase, tyrosinase, dopamine β-hydroxylase. Copper ions are delivered from Cox17 to Sco1 and/or Sco2, and to Cox11, which metalate the Cu_A and Cu_B sites of CCO, respectively. The attained Cu(I) dissociation constants are written beside each protein (Publication I, Fig. S1).

With this high affinity, MTs could take the metal from the cellular copper proteins, but fast copper transfer kinetics between copper chaperones and the target proteins probably overcome the problem. Despite that, MTs and their expression levels play an important regulatory role in the copper trafficking system inside the cell.

To show the applicability of an ESI-MS approach for determination of Zn-binding affinities, the apparent dissociation constants for ZnCox17 complexes were also determined by using DTT as metal-competitive ligand. Zn-binding experiments with human apoCox17 redox forms showed that human apoCox17(2S-S) can bind one Zn(II) ion, whereas apoCox17(0S-S) binds two. Increasing the concentration of DTT resulted in a decrease of the metalated Zn_1Cox17(2S-S) form and an increase of the apoCox17(2S-S) form (Publication III, Fig. 7A). The resulting apparent Zn dissociation constant for the partially reduced Zn_1Cox17(2S-S) form was 0.29 nM. In the case of Zn_2Cox17(0S-S), a two-step demetalation process was observed. With increasing concentrations of DTT, first the Zn_1Cox17(0S-S) form appeared (Publication III, Fig. 7B). The apparent dissociation constant for the first Zn(II) ion was 0.32 ± 0.12 nM (Publication III, Fig. 8A). By further increasing the concentrations of DTT, the second zinc ion was also released from Zn_1Cox17(0S-S) (Publication III, Fig. 7C) with a dissociation constant 0.067 nM (Publication III, Fig. 8B).

CONCLUSIONS

1. Dissociation constant values were determined for almost all intracellular Cu(I)-binding proteins such as, Cox17, Sco1, Sco2, the Cu_A site of the Cox2 subunit, CCS, SOD1, HAH1, ATP7A metal-binding domains 1, 2, 3, 5 and 6, MT-2, MT-3, as well as for GSH and DETC.

2. Our study provides the thermodynamic basis for the kinetic processes that lead to the distribution of cellular copper and shows that affinity gradients drive copper to its cellular destinations.

3. The ESI-MS-based method used is also applicable to other metal ions, and Zn-binding dissociation constants were determined for two Cox17 redox forms.

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SUMMARY

Copper is an essential trace element for cellular organisms. However, intracellular free copper must be strictly limited because of its toxic side-effects. Complex systems for copper trafficking evolved to satisfy cellular requirements while minimizing toxicity. The factors driving copper transfer between protein partners along cellular copper routes are not fully clarified. An important factor in copper transfer from one protein to another is the protein affinity for copper ions, which is quantitatively determined as a dissociation constant (K_d) of the copper-protein complex. The Cu(I) dissociation constants found in the literature differ by several orders of magnitude for the same protein. This variation is very likely to be a result of the variety of technniques used, and even more significantly to be connected with the experimental conditions. In order to characterize the functional cellular copper proteome, corresponding values should be systematically re-estimated under similar conditions. In this study a systematic ESI-MS-based approach was applied to a representative amount of cellular Cu(I)-binding proteins for determination of dissociation constant values of their complexes with Cu(I).

K_d values of intracellular Cu(I) chaperones and enzymes fell into the femtomolar range. Copper chaperones such as HAH1 and Cox17 at micromolar concentrations and GSH at millimolar concentrations have comparable Cu(I)-binding capacities, and therefore constitute an exchangeable cellular copper-binding pool. The copper chaperone CCS has a sevenfold greater Cu(I)-binding affinity than Cox17 and HAH1. Metal-binding domains 1, 2 and 6 of ATP7A have copper-binding affinities three to seven times greater than HAH1, whereas domain 5 has only slightly higher affinity and domain 3 much lower affinity than HAH1. The mitochondrial partners of Cox17, i.e. Sco1 and Sco2, have fivefold greater Cu(I)-binding affinities than Cox17, so that metal transfer occurs from Cox17 to the Sco proteins. The Cu_A site of CcO has fourfold greater affinity for Cu(I) ions than Sco1 and Sco2, thus thermodynamically favoring the transfer of Cu(I) ions from the Sco proteins to the Cu_A site of CcO. SOD1 has tenfold greater Cu(I)-binding affinity than its copper chaperone CCS, so that metal transfer towards the enzyme is thermodynamically favored. There is therefore a distinct Cu(I)-binding hierarchy among Cu(I)-binding proteins, in agreement with the cellular routes of copper delivery, i.e. from chaperones to intermediate copper chaperone proteins and finally to the enzymes, according to the affinity gradient. The role of MTs is intriguing, as because of high Cu(I)-binding affinities in principle they could act as regulators of copper cellular distribution through depriving copper chaperones of their cargo and limiting copper availability to copper enzymes.

Determination of the Zn-binding affinities of Cox17 redox forms shows that the ESI-MS-based method used is also applicable to other metal ions and proteins, for complex analysis of their metal-binding and redox properties.

KOKKUVÕTE

Vask on kõikidele aeroobsetele organismidele ülimalt oluline mikroelement, mis vastutab molekulaarse hapniku kasutamie eest. Samal ajal on vabad vaskioonid äärmiselt toksilised, mis tingib vajaduse vase rakulise jaotuse rangeks regulatsiooniks. Selleks, et rahuldada rakusisest vase nõudlust ning samaaegselt minimaliseerida vase toksilisust, on rakus evolutsiooni käigus tekkinud kompleksed vase transpordi süsteemid. Kuid rakusisese vase transporti mõjutavad faktorid ei ole täielikult teada. Üheks olulisemaks teguriks vase liikumisel valgult valgule on valkude afiinsus vaskioonide suhtes, mille kvantitatiivseks mõõduks on vastava valgu ja vaskiooni vahelise kompleksi dissotsiatsioonikonstant K_d. Kirjanduses toodud Cu(I) komplekside K_d väärtused võivad ühe ja sama valgu kohta erineda mitmete suurusjärkude võrra, mis on tõenäoliselt põhjustatud kasutatud meetodite erinevusest ning eksperimentaalsete tingimuste varieeruvusest. Rakusisese vase proteoomi funktsionaalseks iseloomustamiseks on vajalik vaske siduvate valkude afiinsus määrata ühtse metoodikaga ja sarnastes tingimustes. Käesolevas töös määrati praktiliselt kõigi oluliste rakusiseste Cu(I)-siduvate valkude afiinsused Cu(I) suhtes kasutades selleks ESI-MS metoodikal põhinevat lähenemist.

Rakusiseste Cu(I) šaperonide ja ensüümide K_d väärtused jäid femtomolaarsesse alasse. Vase šaperonide HAH1 ja Cox17 Cu(I) sidumise võime mikromolaarses ja GSH-l millimolaarses kontsentratsioonis on võrreldavad ja nad moodustavad dünaamilise vase reservuaari. Vase šaperonil CCS-il on seitse korda suurem Cu(I) sidumise afiinsus kui Cox17-l ja HAH1-l. ATP7A domeenidel 1, 2 ja 6 on Cu(I) sidumisafiinsused kolm kuni seitse korda kõrgemad kui HAH1-l, samas on domeen 5 afiinsus HAH1 suhtes ainult veidi kõrgem ning domeen 3 afiinsus madalam. Cox17 mitokondriaalsete partnerite Sco1 ja Sco2 afiinsused on viis korda kõrgemad kui Cox17-l, seega on metalliülekanne suunatud Cox17-lt Sco valkude suunas. CCO Cu_A tsentril on neli korda kõrgem Cu(I) afiinsus kui Sco1-l ja Sco2-l, järelikult on Cu(I) transport termodünaamiliselt suunatud Sco valkudelt Cu_A tsentri poole. SOD1-l on kümme korda kõrgem Cu(I) sidumise afiinsus kui tema vase šaperonil CCS-il, seega on metalli ülekanne jällegi suunatud šaperonilt ensüümi poole.

Kokkuvõtteks järeldati, et rakusiseste Cu(I)-valkude seas valitseb nende Cu(I) sidumise afiinsuse osas kindel hierarhia, mis on kooskõlas rakusiseste vase liikumise radadega, s.t. vask liigub šaperonilt kaas-šaperonidele ehk vahepealsetele vasevalkudele ning seejärel ensüümile vastavalt afiinsuse gradiendile. MT-de roll rakus osutus intrigeerivaks, kuna tulenevalt oma kõrgest Cu(I) afiinsusest võivad nad põhimõtteliselt käituda kui rakusisese vase jaotuse regulaatorid, mis on võimelised demetalleerima vase šaperone ja limiteerima vaskioonide kättesaadavust ensüümidele. Käesolevas töös kasutati väljatöötatud ESI-MS

metoodikat ka Zn(II)-ioonide sidumisafiinsuste määramiseks Cox17-e redoksvormidele, mis näitab, et kasutatud metoodika ja lähenemine on rakendatav ka teistele metalliioonidele ja valkudele nende metallisidumis- ja redoksomaduste kompleksseks määramiseks.

PUBLICATION I

Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., **Zovo, K**., and Palumaa, P. „Affinity gradients drive copper to cellular destinations“ (2010) Nature.;465(7298):645-8.

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PUBLICATION III

Zovo K., Palumaa P. “Modulation of redox switches of copper chaperone Cox17 by Zn(II) ions, determined by new ESI MS-based approach” (2009) Antioxid. Redox Signal.;11(5):985-95

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