FEBS Letters 587 (2013) 1902–1910

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Copper chaperones. The concept of conformational control in the metabolism of copper

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ARTICLE INFO

Article history:
Received 25 April 2013
Accepted 6 May 2013
Available online 16 May 2013

Edited by Alexander Gabibov, Vladimir Skulachev, Felix Wieland and Wilhelm Just

Keywords:
Copper chaperones
Copper-thiolate clusters
Copper-binding motifs

ABSTRACT

Copper chaperones compose a specific class of proteins assuring safe handling and specific delivery of potentially harmful copper ions to a variety of essential copper proteins. Copper chaperones are structurally heterogeneous and can exist in multiple metal-loaded as well as oligomeric forms. Moreover, many copper chaperones can exist in various oxidative states and participate in redox catalysis, connected with their functioning. This review is focused on the analysis of the structural and functional properties of copper chaperones and their partners, which allowed us to define specific regulatory principles in copper metabolism connected with copper-induced conformational control of copper proteins.

1. Introduction

Copper is an indispensable microelement for almost all forms of life. Biological evolution has picked copper out as a bioelement mainly for its unique electrochemical properties, however, the same properties, if uncontrolled, may damage the biological systems, and make copper highly toxic. For this reason biological evolution had a two-fold task: to develop molecular systems for biological utilization of copper and to create specific systems for the safe handling of copper ions.

Copper is used as a cofactor by different copper enzymes, some of which are known already for more than a century. However, the systems of copper transport and handling in biological systems have been discovered only around two decades ago [1,2]. Today we do know that cells have highly regulated copper influx and efflux systems and, moreover, copper ions are delivered to the sites of utilization by special proteins called copper chaperones [3]. Copper chaperones are recognized as important components of the copper metabolism, which continues to present itself in ever ever-increasing complexity and also of biomedical importance. Metabolism of copper has direct implications for diverse vital cellular functions like energy production, antioxidant defence, metabolism of iron and peptide hormones, etc., and its disorders are implicated in variety of human pathologies. Disturbed copper metabolism is directly implicated in monogenic Wilson's and Menkes disease [4] and, moreover, copper homeostasis is also disturbed in cancer [5] and in important neurological disorders such as Alzheimer's disease [6] and prion disorders [7]. Understanding the metabolism of copper is therefore important not only for basic life science but is increasingly important also for clinical medicine.

The current review is focused on cytosolic copper chaperones in eukaryotic as well as in some prokaryotic cells. The analysis of the structural and functional properties of various copper chaperones and their partner proteins illuminates the structural heterogenity and multifunctionality of the copper chaperones and allows us to define a new regulatory principle of copper metabolism connected with copper-induced conformational control of copper proteins.

2. Chemical and electrochemical properties of copper ions

Chemical and electrochemical properties of copper ions dictate basic principles of its biological utilization and handling. In the biological systems copper can exist in two ionic forms – Cu(II) and Cu(I), which are dominant in the extracellular oxidative and intracellular reductive environment, respectively. It is relatively easy to handle Cu(II) ions in aqueous solution, as Cu(II) ions are soluble and stabile due to strong hydration. However, Cu(I) ions are weakly hydrated and almost insoluble in water at physiological pH values. Moreover, Cu(I) ions tend to disproportionate to Cu(II) ions and metallic copper unless they are stabilized by complex formation with soft ligands such as sulfur or unsaturated nitrogen. For these reasons special care has to be taken even for solubilization and stabilization of Cu(I) ions in the intracellular reductive environment.

Copper is a redox active metal in aqueous solution. The reduction potential of Cu(II)/Cu(I) couple is 153 mV, however, it is very

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.05.019

susceptible to the complexing ligands and to the geometry of the complex, which makes copper an excellent catalyst for a variety of electron transfer reactions. However, copper ions may also initiate unwanted redox reactions with oxygen derivatives. Most important oxygen derivatives, interacting with copper and other redox-active metal ions like iron are hydrogen peroxide and superoxide radical. The reaction equation for conversion of hydrogen peroxide and superoxide by redox-active metals was presented in 1934 by Haber and Weiss [8] who suggested that highly reactive hydroxyl radicals $HO^{\cdot}$ are produced in the result of the reaction:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{3+}/Fe^{2+} \text{ or } Cu^{+}} O_2 + OH^{\cdot} + OH^{-} \quad (1)$$

Hydroxyl radicals ($OH^{\cdot}$) are utmost reactive oxygen species, reacting at a diffusion controlled rate with practically any type of biomolecules [9]. Haber–Weiss reaction may also produce another type of highly reactive oxygen species (HROS) – a singlet dioxygen $^1O_2(^1\Delta_g)$ [10]. Haber–Weiss equation is the central relationship for understanding the biological metabolism of superoxide and hydrogen peroxide, which are produced in every aerobic cell as the byproducts of aerobic respiration [11,12]. Moreover, Haber–Weiss equation also demonstrates that the metabolism of reactive oxygen species (ROS) is intimately connected with the metabolism of redox-active metals including copper.

2.1. Oxidative stress and antioxidant systems

In all biological systems there is a balanced equilibrium between formation of ROS and their removal by different antioxidative systems. Overproduction of ROS by any pathway or weakening of antioxidative systems leads to the overproduction of HROS and to enhanced oxidative damages in various biomolecules, which forms the molecular background of the oxidative stress [13]. It is convincingly proven that cumulative accumulation of oxidative damages under normal oxidative stress conditions is the main causative factor of normal aging and that oxidative stress induced damages are involved in the pathogenesis of many human neurodegenerative diseases as well as in atherosclerosis, diabetes and other age-related diseases [14–16].

Biological systems have created the entire array of antioxidative systems, which are essential for normal functioning and survival of aerobic organisms. Antioxidative defence is achieved by reduction of the cellular concentration of superoxide and peroxide anions by ubiquitous antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxydase as well as by quenching of HROS by low-molecular antioxidant vitamins and nutrients [9,13]. Moreover, suppression of Haber–Weiss reaction can also be achieved through the lowering of the level of free redox-active metals serving as catalysts of Haber–Weiss reaction, which is a major concern in the metabolism of redox-active metals including copper.

3. Fundamental requirements to the metabolism of copper

To assure effective handling and utilization of copper biological systems have to fulfill all essential requirements arising from the chemical and electrochemical properties of copper ions. It is evident that Cu(I) ions have to be solubilized and stabilized in cellular conditions to prevent precipitation and disproportionation. The main low-molecular ligand for complexation and stabilization of Cu(I) in the intracellular environment is glutathione (GSH), which is present in almost every cell at millimolar concentrations [17]. Besides the solubilization the most important requirement is to suppress harmful Haber–Weiss reaction, which is realized by lowering the cellular pool of free copper ions. As a rule, the biological systems keep the concentration of free cellular copper at an extremely low level. It is estimated that the concentration of free copper in the yeast cell is much lower than a single free copper ion in the volume of the cell [18]. It is evident that such a situation avoids Haber–Weiss reaction, however, ultra-low concentration of cellular free copper may limit the supply of copper to newly synthesized copper proteins and make their activation rather difficult. To assure safe handling and transport of copper ions to essential cellular copper complexes biological systems have invented specific class of proteins called copper chaperones.

4. Copper chaperones

4.1. Bacterial copper chaperones

Bacterial copper metabolism is relatively simple as the bacteria generally do not use copper in the cytoplasm, however, copper is an essential cofactor for cytochrome c oxidase (CCO) residing in the cellular membrane as well as to the periplasmic copper enzymes of some Gram negative bacteria. Some bacteria possess the genes of copper chaperones, products of which might deliver copper to bacterial CCO [19] and to participate in the copper efflux from periplasmic space. Only a small number of bacterial cytosolic copper chaperones are known, which function primarily in copper efflux from the bacterial cells. This review is focused mainly to copper chaperones from *Bacillus subtilis* and *Enterococcus hirae*.

4.2. Bacterial cytosolic copper efflux chaperones

Some bacteria such as *B. subtilis* and *E. hirae* have special copper efflux systems that protect them form the copper toxicity [20,21]. The copper efflux system in *B. subtilis* is composed from a cytosolic copper efflux chaperone denoted CopZ and a copper-transporting ATPase, denoted as CopA [20]. Such a two-component copper efflux system is the simplest example of chaperoned copper delivery, and similar systems do exist also in the eukaryotic cells. *E. hirae* proteome contains also the copper efflux chaperone CopZ (EhCopZ), however, it contains two copper ATPases - CopA and CopB, which are suggested to participate in copper influx and efflux, respectively. Moreover, EhCopZ also delivers the copper ions to copper-sensitive transcription factor CopY [21].

4.2.1. Structure of bacterial copper efflux chaperones

Bacterial cytosolic copper chaperone CopZ is a small protein composed from approximately 70 residues containing a metal-binding motif MXCXXC. Apo-CopZ proteins are monomeric and fold into a ferredoxin-like fold (ßαßßαß), where four ß-strands and two α-helices form a double sandwich. The MXCXXC metal-binding motif is located in the loop connecting the first ß-strand with the first α-helix [22]. Both chaperons can exist in several metalloforms, which retain the ferredoxin-like fold but expose different metal-binding stoichiometry and form different oligomers. In the presence of external free thiol groups the CopZ protein binds one Cu(I) ion and is in the monomeric form [20,23]. The metal-binding site in Cu_1CopZ composed from two Cys is exposed to the solvent and can bind external thiols like GSH or DTT [20,24].

In the absence of environmental free thiol groups BsCopZ undergoes a metal-induced dimerization leading to various metalloforms with varying metal-binding stoichiometry [23]. There is evidence that dimeric CuBsCopZ contains a dicopper-tetrathioate cluster at the protein–protein contact interface [25]. However, the crystallization of dimeric Cu_2BsCopz yielded to a dimeric CopZ metalloform containing tetracopper Cu(I) cluster at the protein–protein contact interface [26]. Dimeric EhCopZ, assembled at the high protein concentration in the absence of thiols binds only a single Cu(I) ion [24], which is most probably shared by two monomers.

4.2.2. Copper efflux chaperone–target interaction

BsCopZ interacts with the Cu-ATPase CopA (BsCopA), residing in cellular membrane and responsible for the active transport of copper out from the cell [27]. The N-terminal cytosolic part of BsCopA contains two copper-binding domains (CuBD) that are structurally similar to CopZ. The individual CuBDs of BsCopA interact with BsCopZ and form heterodimeric metal-transfer complexes using their MXCXXC metal binding motifs [28]. However, the NMR structure of the whole N-terminal region of BsCopA demonstrates, that the two CuBDs are folded into a globular structure [27]. According to the proposed model Cu_1BsCopZ interacts via protein–protein interaction with individual metal-binding sites on the N-terminal globule of BsCopA and transfers the metal ion in metal-bridged protein–protein complex by sequential ligand exchange reaction [27]. The protein–protein interactions in the complex are favored by the electrostatic interactions between the negative surface charges of CopZ and the positive surface charges of CopA [27,28]. However, the experiments with *Archaeoglobus fulgidus* CopZ (AfCopZ) and the functional CopA (AfCopA) protein demonstrate that AfCopZ can donate the copper directly into the membrane bound Cu(I)-binding site for the translocation through the membrane [29]. CopZ also donates Cu(I) to the CuBD-s of CopA, which most probably has a regulatory role [29].

The studies of bacterial CopZ proteins have demonstrated that depending on the environmental conditions the copper efflux chaperones may exist in different monomeric and dimeric metalloforms and form ternary complexes with external thiols. It is suggested that the physiological metalloform of CopZ is a Cu1CopZ monomer, where GSH completes the coordination sphere of Cu(I) ion and prevents homodimer formation [25]. Dimeric CopZ might be a dead-end product in the metal transfer [25], however, this complex may also be connected with the scavenging of copper ions into safe solvent shielded complexes.

5. Eukaryotic copper chaperones

The copper metabolism in eukaryotes differs from that in prokaryotes. The most principal difference is connected with the utilization of copper in the cytoplasm as well as in various cellular organelles such as mitochondria and endoplasmatic reticulum [3]. To guarantee the supply of copper to the intracellular copper proteins, eukaryotes have invented a high-affinity copper influx system, denoted as Ctr-transporters. Under the copper-limiting conditions the Ctr transporters function in an energy-independent mode and transport Cu(I) ions into the cytoplasm [4,30].

In addition to this specific copper influx system, eukaryotic cells have also invented many new copper chaperones assuring the delivery of copper to the new sites of utilization located in cytoplasm, endoplasmatic reticulum and mitochondria.

5.1. Eukaryotic copper efflux chaperones

Eukaryotic organisms possess the homologs of the bacterial copper efflux chaperone and Cu-ATPases, which confirms the prokaryotic origin of the eukaryotic copper efflux system. However, during the evolution this system has been sufficiently sophisticated to accommodate the transport of copper into multiple destinations, such as endoplasmatic reticulum, extracellular space and cytosolic vesicles. The transport of copper to various locations is realized by the same two-component system, composed from copper efflux chaperone and its partner Cu-ATPase, however, the copper destination depends of the localization of the Cu-ATPase [31]. At the limiting or normal copper concentrations the eukaryotic Cu-ATPases are localized in the membranes of trans-Golgi network and deliver copper from the cytosol to the trans Golgi for the insertion into *de novo* synthesized and secreted copper enzymes. In the result of elevated concentration of copper or other stimuli, Cu-ATPases will relocalize into the cellular or vesicular membranes and ensure the copper efflux from the cytoplasm into the extracellular space and/or into the cytoplasmic vesicles [31–33].

5.1.1. Structure of eukaryotic copper efflux chaperones

The eukaryotic copper efflux chaperones denoted Atx1 in yeast and Hah1 or Atox1 in mammals are homologous and structurally similar to the bacterial copper efflux chaperone CopZ by displaying a ferredoxin-like fold with the MXCXGC metal-binding motif in the first loop [34,35].

The eukaryotic copper efflux chaperones can also form multiple monomeric and dimeric metalloforms. In the absence of free thiol groups in the medium Atx1 binds one Cu(I) ion, which is linearly coordinated by two Cys residues [34]. The metal-binding site in Cu_1Atx1 is, however, buried and the eukaryotic chaperon has a lower tendency to form ternary complexes with exogeneous thiols than its prokaryotic counterpart [34]. On the contrary, the metal-binding site of Cu_1Hah1 is exposed to the solvent and Cu_1Hah1 can easily form ternary complexes with exogeneous thiols [35,36].

The crystal structure of the dimeric metalloform of Hah1 exposes a metal-bridged dimer $Cu_1(Hah1)_2$, where Cu(I) is bound in a distorted tetrahedral metal-binding site consisting of four Cys residues [37]. In the dimeric $Cu_1(Hah1)_2$ the copper ion is practically inaccessible to the environment [37] and therefore the role of homodimers might be the shielding of copper ions from the oxidants present in the environment.

5.1.2. Structure and metal-binding properties of eukaryotic Cu-ATPases

The partners of eukaryotic copper efflux chaperones in the cells are eukaryotic Cu-ATPases, which general architecture is similar to the bacterial homolog CopA. The single copper ATPase present in the yeast is denoted as Ccc2 and this protein contains two N-terminal CuBDs, however, the mammals have two tissue-specific Cu-ATPases – ATP7A and ATP7B, which contain as much as six CuBDs at their N-terminus [3]. The latter two proteins are affected in the case of human genetic copper-transport disorders Menke's and Wilson's disease and are called also Menkes and Wilsons disease proteins [38].

All CuBDs of eukaryotic Cu-ATPases are also folded into a ferredoxin-like fold [39]. Two CuBDs of yeast Ccc2 have been structurally characterized and only the first of them had a well-defined 3D-structure, whereas the second domain was less structured [40]. The structural studies of the constructs containing multiple CuBDs of ATP7A demonstrate that the domains are individually folded and do not interact with each other and do not form a compact globular structure [41–43].

The individual CuBDs of eukaryotic Cu-ATPases have metal-binding properties similar to the bacterial copper efflux chaperones. However, the multiple copper-binding domains do not function separately in the metal binding but cooperate with each other. Two N-terminal CuBDs of yeast Ccc2 containing six Cys residues can bind four Cu(I) ions, whereas the process is accompanied by the destruction of ferredoxin-like fold [37]. The six CuBDs of mammalian CuATPases also demonstrate positive cooperativity in the copper binding [44] and form tetracopper-thiolate clusters [33,45]. It is suggested that the copper-thiolate clusters may be involved in the relocalizaton of copper ATPases [33]. The relocalization of ATPases is a multifactorial process that is defective in the cases of some mutations linked with Menke's and Wilson's disease.

5.1.3. Interaction of eukaryotic copper efflux chaperones with target

Eukaryotic copper efflux chaperones form copper-induced protein–protein complexes with individual CuBDs of Cu-ATPases

[46] and it is suggested that the metal transfer may occur by a sequential ligand exchange reaction like in the prokaryotic system [47–49]. According to this mechanism the copper-binding sites function independently of each other, which may be valid at low concentrations of copper ions, however, the metal-transfer model with a single shared metal ion is definitely invalid at elevated copper concentrations where the multiple CuBDs of Cu-ATPase form copper-thiolate clusters. The role of copper efflux chaperones in the formation of copper-thiolate clusters as well as the role of the clusters in the copper transport by CuATPases is unknown.

5.2. Copper chaperone for Cu,Zn-SOD

Cu,Zn-SOD is the only copper enzyme, which is located in the cytoplasm and, besides this location, it is also present in the mitochondrial intermembrane space (IMS). The delivery of copper to Cu,Zn-SOD in both locations is conducted by a specific copper chaperone denoted as Ccs [3,50].

5.2.1. Structure and functions of copper chaperone for Zn, Cu-SOD

Ccs consist of two domains and a short disordered C-terminal fragment, which contains the conserved CXC motif [39]. The structure of the first domain of Ccs is similar to that of copper efflux chaperones, it is folded to a ferredoxin-like fold and contains a MXCXXC metal binding motif [51]. The second domain of Ccs is highly homologous to Cu,Zn-SOD [51,52] and is responsible for the interaction with the target protein. The crystal structures of the homodimeric complexes of Ccs [51] and heterodimeric Ccs-SOD complex [53] confirm the essential role of protein–protein interactions in the functioning of Ccs.

The C-terminal disordered fragment of Ccs contains a conserved CXC motif [54], and has, a highly specific role in the maturation of Cu,Zn-SOD by catalyzing the formation of the conserved disulfide bond in the Cu,Zn-SOD molecule, which is important for the protein stability [55]. Thus, the copper chaperone for Cu,Zn-SOD functions also as a protein sulfydryl oxidase. Interestingly there are some species-specific differences in the functioning of Ccs. Ccs is absolutely necessary for the metallation and the oxidation of the yeast Cu,Zn-SOD, whereas the Cu,Zn-SOD in human and in *Caenorhabditis elegans* can be oxidized and metallated in the absence of Ccs most probably with the assistance of GSH [3].

5.2.2. Metal-binding properties of copper chaperone for Zn, CuSOD

The metal-binding properties of Ccs are different from those of copper efflux chaperones. The individual domain I of Ccs binds one Cu(I) ion and the individual domain II also binds one Zn(II) ion, however, in the presence of DTT the domain II and the C-terminal unstructured region do not bind Cu(I) [56]. At the elevated copper concentrations the domain I does not bind metal ion independently but forms a dinuclear copper-thiolate cluster together with the C-terminal CXC motif [57]. A similar cluster exists also in the dimeric Ccs [58]. In slightly different conditions the dimeric Ccs can also form tetracopper-thiolate clusters [57–59].

6. Transport of copper to mitochondrial CCO

CCO, which resides in the mitochondrial inner membrane, plays a crucial role in the aerobic energy metabolism of all eukaryotic cells. CCO transfers electrons from cytochrome-c to the molecular oxygen with the assistance of its two hemes and three copper ions that are located in binuclear Cu_A and mononuclear Cu_B sites respectively [60]. CCO supplementation with copper is a vital task of enormous importance, however, this task is also a rather complicated one as copper has to be transported from the cytosol to the mitochondria and incorporated into two different subunits of CCO. At the moment, at least six proteins – soluble proteins Cox17, Cox19, Cox23 and mitochondrial inner membrane proteins Sco1, Sco2 and Cox 11 – can be classified as copper chaperones for CCO [3].

6.1. Delivery of copper to Cu_B site of CCO by copper chaperone Cox11

The Cu(B) site is located in the subunit Cox1, which is the biggest and most hydrophobic protein subunit of the CCO complex [60]. The formation of the Cu_B site, which is buried 13 Å below the membrane surface, depends on the copper chaperone Cox11 that present in all eukaryotes and in some Gram-negative bacteria [19]. The yeast Cox11 is a 34 kDa protein composed from a N-terminal region located in the mitochondrial matrix, a single transmembrane helix anchoring Cox11 into the inner mitochondrial membrane and a C-terminal metal-binding domain located in IMS. The structure of the soluble domain is known only for bacterial Cox11. Cox11 has a β-immunoglobuline-like fold and its copper-binding site is composed of the two Cys residues in the CFCF motif [61]. The functional form of Cox11 is dimer that binds two Cu(I) ions in a dinuclear Cu_2S_4-type of cluster located at dimeric interface [61,62].

The mechanism of copper transfer from Cox11 to Cu_B site of CCO is largely unknown. The metal transfer through the protein–protein interaction and ligand exchange between the soluble domain of Cox11 and Cox1 is hampered as the Cu_B site is buried below the membrane surface [61]. It is suggested that the Cu_B site is co-translationally formed by a transient interaction between copper-loaded Cox11 and the nascent Cox1 protein in the IMS [63].

6.2. Delivery of copper to Cu_A site of CCO

The dinuclear Cu_A site of CCO is located in the subunit Cox2, which is exposed to mitochondrial IMS [60]. The metallation of the Cu_A site is apparently the most complicated task of copper delivery as it requires the highest number of assisting proteins: Cox17, Sco1, Sco2, Cox19 and Cox23 are assumed to be involved in this process.

6.2.1. Cox17

Cox17 is a small 7 kDa protein, containing six conserved Cys residues. Cox17 is functioning in the mitochondrial IMS [64], however, it can be also found in the cytosol. Cox17 lacks the mitochondrial targeting sequence and it is transported into the mitochondrial IMS by the assistance of Mia40 and Erv1 proteins during the oxidative folding [65]. In vitro experiments demonstrate that indeed the mammalian Cox17 may exist in three oxidative states – in a fully oxidized (3 disulfides), in a partially oxidized (2 disulfides – $Cox17_{2S\text{-}S}$) and in a fully reduced state [66]. It is feasible that Cox17 exists in IMS in a partially oxidized $Cox17_{2S\text{-}S}$ form, which might be essential for its retention in IMS [67].

The different oxidative forms of Cox17 display different metal binding properties: the fully reduced Cox17 binds cooperatively four Cu(I) ions into a solvent-shielded tetracopper-thiolate Cu_4S_6-type of cluster. The $Cox17_{2S\text{-}S}$ form can bind only one Cu(I), whereas the fully oxidized Cox17 does not bind metal ions [66].

The 3D-structures are known for the partially oxidized yeast and human $Cox17_{2S\text{-}S}$ forms, which display an unstructured N-terminal region followed by two helixes, connected to each other by two disulfide bonds [68,69]. Such a domain is called coiled-coil-helix-coiled-coil-helix (CHCH) and its formation is enabled by two consecutive CX_9C motifs. The metal-binding site in $Cu_1Cox17_{2S\text{-}S}$ is composed from two vicinal Cys residues located just before the first α-helix [68,69].

The functioning of Cox17 depends on the oxidative state of Cox17 in different cell compartments. Cu_4Cox17 may be present in the cellular cytosol, however, its existence and cellular role is

unknown [67]. The partially oxidized Cu_1Cox17_{2S-S} is dominant in IMS [67], and it participates in the metal transfer to Cox11 and Sco1 proteins [70,71]. Besides the metal transfer reaction Cu_1-$Cox17_{2S-S}$ can also participate in the reduction of oxidized Sco1 protein [72], which makes possible the metal transfer in the oxidizing conditions of IMS.

6.2.2. Sco1& Sco2

Sco1/2 genes are present in some prokaryotic and in all eukaryotic genomes [19]. Sco proteins are anchored to the mitochondrial inner membrane by a single transmembrane helix and contain a soluble metal-binding domain located in IMS. Sco1 interacts with Cox2 subunit of CCO and may participate in the delivery of copper to Cu_A site of CCO [73].

The apo forms of bacterial (*B. subtilis*), yeast and human Sco1 as well as human Sco2 are folded into a thioredoxin fold and contain 2 Cys residues in a CXXXCP motif [71,74–76],This motif has a catalytic role in the thioredoxins that are reducing protein disulfides. Sco binds copper(I), copper(II) and other metal ions which supports its role as a copper chaperone. The metal-binding sites of Sco1 and Sco2 are composed of two Cys in CXXXCP motif and a conserved distant His residue [71,74,76]. It has been suggested that Sco1 can also function as a thioredoxin [71,74]. Genome based investigations and experiments with bacterial Sco protein support the dual role of Sco proteins in the maturation of CCO [77,78]. The mechanism of Sco1 action in the maturation of eukaryotic CCO as a copper chaperone and a thioredoxin awaits for further investigations.

6.2.3. Cox19 & Cox23

Cox19 and Cox23 are essential for the activation of CcO [79,80] which indicates that they have non-overlapping functions with Cox17 and Sco proteins. Cox19 and Cox23 are small soluble proteins containing four conserved Cys residues in two CX_9C motifs [69]. It cannot be excluded that all four Cys residues of Cox19 and Cox23 are necessary for the protein translocation into the intermembrane space where they are forming disulfide bridges during the oxidative folding. However, it has been demonstrated that the recombinant Cox19 binds 1 mol eq of Cu(I) per monomer and exists as a dimeric protein, whereas Cox19 isolated from the mitochondrial intermembrane space contains variable quantities of copper [81]. The function and partners of Cox19 and Cox23 are largely unknown.

7. Characterization of copper chaperone protein family

The available structural and functional data on bacterial and eukaryotic copper chaperones are summarized in Table 1. It is seen from the Table 1 that the copper chaperones play highly specific and unique roles in the delivery of copper to different copper proteins as diverse as membrane-embedded copper transporters, soluble copper enzymes and electron transfer complexes located in mitochondrial IMS or buried into the mitochondrial inner membrane.

7.1. Structural diversity

The family of copper chaperones is structurally heterogeneous. There are examples of single-domain and multidomain, monomeric and dimeric as well as soluble and membrane anchored proteins in many combinations. Multiple different folds have been used for building up the copper-binding domains (Fig. 1), which apparently depends on the metal transfer mechanism. The most common fold of copper efflux chaperones, CuBDs of Cu-ATPases and domain I of the copper chaperone for Cu,Zn-SOD is the ferredoxin-like fold, which enables the formation of electrostatically favorable and transient metal-bridged protein–protein complexes. The simplest fold among copper chaperons is the coiled-coil-helix-coiled-coil-helix (CHCH) motif found in $Cox17_{2S-S}$, and most probably also in Cox19 and Cox23. The thioredoxin fold in Sco1 proteins may possess two functions: copper transfer and redox catalysis. The immunoglobulin-like fold in Cox11 may be necessary for the creating of a highly hydrophobic copper-binding site in dimeric Cox11.

7.2. Functional diversity of copper chaperones

Copper chaperones display a variety of copper transfer mechanisms, which include the formation of metal-bridged heterodimeric complexes in the case of copper efflux chaperones, heterodimeric protein–protein complexes in the case of Ccs, a transient metal-transfer complex in the case of Cox17, as well as interaction of Cox11 with a nascent polpeptide chain.

Moreover, many copper chaperones are multifunctional proteins and the major secondary function of copper chaperones is connected with redox catalysis. The best known example is the copper chaperone for Cu,Zn-SOD, which has an important sulfhydryl oxidase role in the maturation of Cu,Zn-SOD. The participation of Sco proteins having characteristic thioredoxin fold in redox catalysis is also highly probable. Redox processes are also important for the functioning of Cox17 that exists in three oxidative states. Cox19 and Cox23 proteins may even not be involved in the direct metal transfer but they can be involved in redox processes, assisting protein relocalization or copper delivery.

7.3. Structural plasticity of copper chaperones

The results summarized in Table 2 indicate that all copper chaperones can form multiple different metalloforms. This behavior does not fit into the paradigm of common metalloproteins, characterized by the occurrence of a single distinct metalloform. Besides the common mononuclear metal-binding forms can many copper chaperones form multinuclear copper-thiolate clusters (Table 2). The dicopper-tetrahiolate clusters occur in dimeric copper efflux chaperones, in dimeric Ccs and Cox11. In these structures the metal ions are bridging two monomers and contribute to the stability of the dimer, whereas the copper ions themselves are shielded from the environment. The tetracopper-thiolate cluster, which is formed in dimeric bactrerial CopZ, fully reduced Cox17, dimeric Ccs, and also in MBD-s of eukaryotic Cu-ATPases and in the C-terminal part of yeast copper influx transporter Ctr1 is an another common motif [82]. Based on the results of computational analysis it has been suggested that the formation of dicopper-tetrathiolate and tetracopper-hexathiolate clusters is energetically favorable in the presence of a sufficient number of thiolate ligands and does not depend on the particular protein sequence [83].

The formation of polycopper-thiolate clusters at elevated copper concentrations may have many biological consequences. The polycopper-thiolate clusters are well characterized structural motifs in metallothioneins (MT). MTs are low molecular weight Cys-rich proteins, present in all eukaryotic organisms and in some prokaryotes [84]. Yeast MT binds copper into a octacopper-decathiolate (Cu_8S_{10})-type of cluster, which is the largest known oligonuclear Cu(I)-thiolate cluster in the living organisms [85]. MTs in the higher organisms bind copper into two copper-thiolate clusters whereas the tetracopper-thiolate clusters are the most common ones [86,87]. Numerous data confirm that MTs participate in the safe intracellular copper storage and protect cells against the copper toxicity [84], which indicates that the solvent-shielded Cu(I)-thiolate clusters serve as biologically safe complexes, where the copper ions are redox silenced and unable to catalyze Haber–Weiss reaction. The polycopper-thiolate clusters are

Table 1
Structure and functions of copper chaperones.

Copper chaperone Specie/Occurrence	Domains Fold Type Oligomeric state	Metal-binding sequence, motif(s)	Target	Primary function	Other functions
CopZ *Bacillus subtilis*	One domain, Ferredoxine-like Monomer/ Dimer	MXCXXC	Cu-ATPase CopA (2 N-terminal CuBDs)	Metallation of Cu-ATPases	Unknown
Atx1 *S. cerevisiae*	One domain, Ferredoxine-like Monomer/ Dimer	MXCXGC	Cu-ATPase Ccs2 (2 N-terminal CuBDs)	Metallation of Cu-ATPases	Unknown
Hah1 or **Atox1** Higher organisms	One domain, Ferredoxine-like Monomer/ Dimer	MXCXGC	Cu-ATPases ATP7A and ATP7B	Metallation of Cu-ATPases	Unknown
Ccs Eukaryotes	Two domains + irregular part Ferredoxin-like, SOD-like, unstructured Monomer/Dimer	MXCXXC in domain1 & CXC in C-terminus	Cu,Zn SOD	Metallation of Cu,Zn-SOD	Catalysis of disulfide formation in Cu,Zn-SOD
Cox11 Eukaryotes & some prokaryotes	N-terminal part + TMH + one domain Immunoglobulin-like Dimer	CFCF	Cox1 subunit of CCO	Metallation of Cu_B site of CCO	Unknown
Cox17 Eukaryotes	One domain, Cox 17_{2S-S} - CHCH-domain Cox 17-metal cluster Monomer	CCinCox17_{2S-S} 6 Cys in Cox 17	Sco1, Cox11	Metallation of Sco 1 andCox11	Reduction of oxidized Sco1
Sco1 Eukaryotes & some prokaryotes	Soluble domain + TMH Thioredoxin-like Dimer	CXXXCP + His	Cox2 subunit of CCO	Metallation of Cu_A site of CCO	Thioredoxin. Reduction of oxidized Cu site of CCO A
Sco2 Eukaryotes & some prokaryotes	Soluble domain + TMH Thioredoxin-like Dimer	CXXXCP + His	Unknown	Metallation of Cu site of CCO?	Thioredoxin?
Cox19 & **Cox23** Eukaryotes	One domain, CHCH (by homology) Monomer?	Unknown	Unknown	Unknown Copper transfer?	Unknown

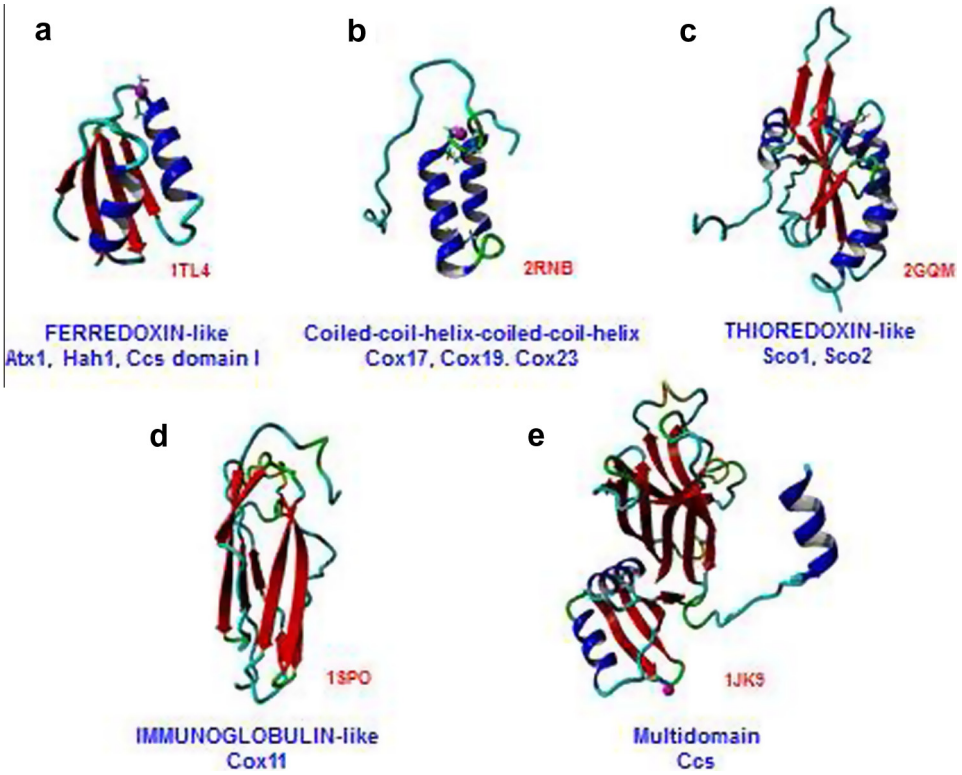

Fig. 1. Structure of copper chaperones. (a) Ferredoxin-like fold; (b) coiled-coil-helix-coiled-coil-helix (CHCH) fold; (c) thioredoxin-like fold; (d) immunoglobulin-like fold; (e) multidomain structure of Ccs.

particularly suited for this function as they can form fast and in a cooperative manner, which enables rapid response to the elevated concentrations of copper ions and moreover, polycopper clusters provide a high metal-binding capacity. The formation of solvent-shielded polycopper-thiolate clusters with copper chaperones and their partners may scavenge Cu(I) ions from the environment and protect cells against toxicity of free copper ions similarly.

Besides the protective function, the formation of copper-thiolate clusters may also modify the functioning of copper chaperones. In the case of Cox 17 and Ccs the formation of polycopper clusters may switch the function of the protein from a copper chaperone to the metal-scavenging molecule, which is beneficial at elevated copper concentrations, where the target copper proteins are already saturated with copper. The copper clusters at the N-terminal

Table 2
Metal-binding motifs in copper chaperones and copper proteins.

Copper chaperone or copper protein	Metal-binding sequence	Existing metalloforms	Metal-binding motif(s)
Copper efflux chaperones CopZ, Atx1 Hah1 or Atox1 & **CuBDs of Cu-ATPases**	MXCXXC	1. Cu Protein Monomer 2. Cu ProteinGSH Monomer 3. Cu Protein Dimer 4. Cu Protein Dimer	1. $Cu(I)_1Cys_2$ 2. Cu(I)1Cys2GSH 3. $Cu(I)_1Cys_3$ 4a. Cu(I) S -type of cluster 4b. $Cu_4S_4His_2$ -type of cluster
Copper chaperone for Cu,Zn-SOD (Ccs)	MXCXXC in Domain 1 and CXC in irregular part	1. Cu_1Ccs Monomer 2. $Cu_2(Ccs)_2$ Dimer 3. Cu_4 $(Ccs)_2$ Dimer	1. $Cu(I)_1Cys_2$ 2. $Cu(I)_2Cys_4$ 3. Cu_4S_6-type of cluster
Cox11	CFCF	1. Cu_2 Cox11 Dimer	$Cu(I)_2S_4$ -type of cluster
Cox17	1. 6Cys in Cox17 2. CC in $Cox17_{2S-S}$	1. Cu_4 Cox 17 Monomer 2. Cu_1 $Cox17_{2S-S}$ Monomer	1. $Cu(I)_4S_6$-type of cluster 2. $Cu(I)_1Cys_2$
Sco1/Sco2	CXXXCP + His	Cu Sco1 Monomer	$Cu(I)_1Cys_2$+His
Multiple CuBDs of Cu-ATPases	2 CXXC motifs 2 Cys in 2 CuBDs 6 CXXC motif in 6 CuBDs	1. Cu_4(Ccc2) 2. Cu_4 ATP7A	1. Cu_4 S_6-type of cluster 2. Cu_4 S_6-type of cluster
Copper influx transporter Ctr1	6 Cys residues in C-terminus	Cu_4Ctr1	Cu_4S_6 -type of cluster
Metallothioneins	Multiple Cys residues	Cu_8 Cup1 (yeast MT) Cu_4 MT-1/2/3	Cu_8S_{10}-type of cluster Cu_4S_6-type of cluster

region of eukaryotic Cu-ATPases may direct their relocation, however, the clusters may play also an active role in the metal transport.

8. Summary

8.1. Concept of conformational control in copper metabolism

The conformational plasticity in copper binding appears to be a common feature of copper chaperones and their partners suggesting that this feature might provide an additional mechanism for the control and regulation of copper metabolism. The suggested mechanism has an advantage over the common transcriptionally controlled regulation mechanisms because the putative concentration-driven switch in the functioning of copper chaperones and their partners together with the formation of safe copper-thiolate clusters at the elevated copper concentrations provides a much faster feedback to increased copper concentrations and lowers the risk of free copper-induced oxidative damage. In evolution the copper-thiolate clusters have been invented in bacteria but they are ubiquitous in the eukaryotic cells and there is a tendency of more extensive presence of these structural elements in higher organisms. Such a tendency confirms further that the polycopper-thiolate clusters serve as essential structural elements for safe biological handling of copper ions and their more extensive utilization by higher organisms might provide additional cellular defense against copper-catalyzed Haber–Weiss reaction, necessary for the functioning and survival of higher and long-living organisms.

Acknowledgment

We acknowledge Estonian Science Foundation grant No. 8811 for financial support.

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