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REVIEW

Cellular copper distribution: a mechanistic systems biology approach

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Abstract Copper is an essential but potentially harmful trace element required in many enzymatic processes involving redox chemistry. Cellular copper homeostasis in mammals is predominantly maintained by regulating copper transport through the copper import CTR proteins and the copper exporters ATP7A and ATP7B. Once copper is imported into the cell, several pathways involving a number of copper proteins are responsible for trafficking it specifically where it is required for cellular life, thus avoiding the release of harmful free copper ions. In this study we review recent progress made in understanding the molecular mechanisms of copper transport in cells by analyzing structural features of copper proteins, their mode of interaction, and their thermodynamic and kinetic parameters, thus contributing to systems biology of copper within the cell.

Keywords Copper trafficking · ATP7A · ATP7B · Cytochrome *c* oxidase · Superoxide dismutase · Copper chaperone

Introduction

Copper is an extremely important trace metal in biological systems as it is essential for the life of eukaryotic organisms [1]. It is a cofactor in a variety of proteins and enzymes present in multiple cellular locations. The size of the copper proteome is however limited, generally less than 1% of the total proteome in both eukaryotes and prokaryotes [2]. The ability of copper to adopt distinct redox states by cycling between the oxidized Cu(II) and reduced Cu(I) forms makes it a suitable cofactor for a wide range of catalytic functions in metal-binding enzymes. In humans, it is distributed throughout the body and participates in a range of physiological processes including central nervous system functions, connective tissue and blood vessel development, pigmentation, reactive oxygen species detoxification, synaptogenesis and mitochondrial functions [1, 3]. The same redox properties paradoxically lead to cellular oxidative damage when copper is found in excessive concentrations. Copper participates in reactions that lead to production of highly reactive oxidative species (such as hydroxyl radicals), which can have devastating effects in cells, including DNA damage and oxidation of proteins and lipids [4]. Copper toxicity also results from the high affinity that Cu(I) and Cu(II) exhibit for a range of protein sites, with cysteine, methionine and histidine side chains as potential ligands, resulting in the displacement of native metal ions from their active sites, as well as in the misfolding of proteins. However, the proteins newly produced by ribosomes need to acquire the copper ion in a quantitative, efficient and rapid way. Thus, copper intake, distribution, utilization and excretion must be tightly regulated, and copper ions must be bound to proteins rather than "free" throughout this process [5, 6]. It is through protein-protein recognition processes that correct delivery to Cu-dependent enzymes and subsequent removal from tissues and the body to avoid toxicity is ensured. Several highly sophisticated systems of cellular copper transport have been discovered in both eukaryotic and prokaryotic cells [5, 7]. In these copper trafficking systems, copper is transported across the cell membrane and once inside the cell it is bound by chaperone proteins responsible for its transfer to the final destination or to any

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intermediate location. The first is a copper requiring protein or enzyme, while the second is another copper-trafficking protein, which transports the copper across a membrane into a cell compartment or out of the cell if copper concentration exceeds the optimum level. Another mechanism that cells use to avoid high toxic copper levels relies on copper chelation through cysteine-rich proteins (metallothioneins) that bind the metal within multi-nuclear clusters [8].

It is important to know the nature of all the players in these processes, their oxidation and metallation states, and how they interact in order to describe how the various pathways operate and to understand the factors determining copper transport processes, their selectivity and specificity. Bioinformatic analysis of the genomes should be performed to search for these proteins in the organism under investigation, and a paralog search should be performed to analyze correlations, similarities and divergences. The proteins involved in these processes need to be framed in a cellular context taking into consideration all the species present in the cellular compartment where the various processes take place. Indeed, proteins are not isolated and the various processes, if they share any one of the players, would influence each other.

Knowledge of the structural features, the mode of interaction among partners and the thermodynamic and kinetic parameters can contribute to the development of models for describing a system and how it functions. This would be a contribution to systems biology and could be defined as mechanistic systems biology based on its molecular and functional aspects. In this review we examine the best characterized processes responsible for copper transport and homeostasis.

Copper import into the cytoplasm

In eukaryotic organisms there is a stringent requirement for copper import into the cytoplasm not only for the complete maturation of cytoplasmic cuproproteins, but also for enzymes assembled in organelles such as cytochrome *c* oxidase in mitochondria and tyrosinase and ceruloplasmin in the Golgi apparatus. The mechanisms of copper uptake in an eukaryotic cell are tightly regulated and involve specific high-affinity plasma membrane copper transporters or low-affinity permeases [9, 10].

Genetic experiments with baker's yeast, *Saccharomyces cerevisiae,* resulted in the identification of the first plasma membrane-associated high-affinity copper transport protein (Ctr), denoted Ctr1 [11]. Yeast cells lacking a functional Ctr1 gene are indeed defective in mitochondrial oxidative phosphorylation due to their inability to load copper into cytochrome oxidase, and as such, they fail to grow when provided with non-fermentable carbon sources as their only source of carbon [12, 13]. Since that time, genes encoding the Ctr protein family have been isolated from mice, humans and other organisms because of their significant sequence homology and remarkable functional conservation. All members of the Ctr family of copper transporters have three transmembrane helices, a hydrophilic amino terminus typically rich in methionine residues outside of the membrane, and a number of Cys/His conserved residues at the C-terminus in the cytoplasm (Fig. 1) [9]. Ctr1 acts on reduced copper [Cu(I)] rather than on the oxidized metal ion, [Cu(II)]. This preference for Cu(I) is supported by (1) the requirement of yeast cell-surface metalloreductase activity for high-affinity copper uptake; (2) by the stimulation of copper uptake in yeast and mammals by external reductant; (3) by the ability of silver, a Cu(I) mimetic, to strongly compete for copper uptake stimulated by Ctr1 [14–16].

Very recently, the 3D-structure of human Ctr, solved by electron crystallography to an in-plane resolution of 7 Å, revealed that overall, the transporter conforms to the design of a "traditional" ion channel in which a membrane-spanning pore is created by a symmetrical homotrimer [17]. Located outside the membrane, additional electron densities formed by the N-terminal and the intracellular domains of human Ctr1 are observed, suggesting that the extramembrane regions of Ctr1 provide important structural elements regulating copper access to and from the pore. Correspondingly, previous studies established the importance of extracellular Met-rich motifs in the N terminus of Ctr-proteins for copper uptake [18, 19] and that

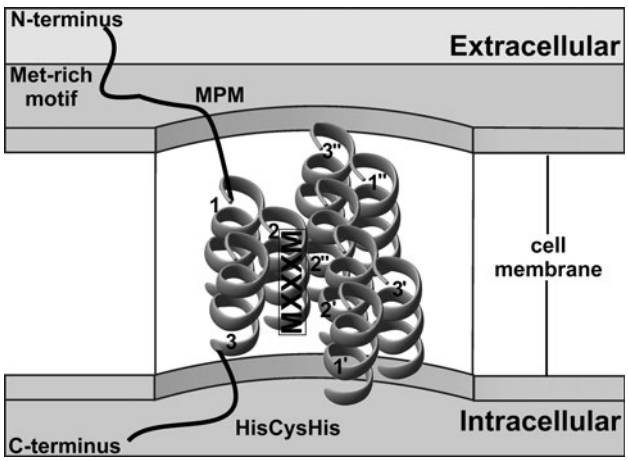

Fig. 1 Schematic representation of the topology of human Ctr1. Trans-membrane (*TM*) segments of the same monomer are labeled with the number of the corresponding trans-membrane segments in the sequence (1 = TM1, 2 = TM2, 3 = TM3). The copper-binding MxxxM-motif in TM2 is labeled only in one monomer. Met-rich motif refers to the multiple metal-binding residues present in the N-terminal tail. MPM- and HCH-binding motifs at the N- and C-termini are indicated, respectively

Cys-containing clusters have been implicated in metal binding at the C terminus on the cytoplasmatic side [19, 20]. Although the Met motifs at the N-terminus are not essential for Cu(I) transport, they are important for Cu(I) uptake in vivo at highly limiting extracellular copper concentrations [19]. A strictly conserved MxxxM-sequence motif on the second transmembrane helix (TM2) of Ctr-proteins is mandatory for copper uptake providing ligands for Cu(I) binding during the passage through the membrane channel [18, 19]. In support of this idea, the TM2 M154C variant of human Ctr1 can form disulfide-linked dimers, indicating that the two methionine residues are in close proximity within the channel [17]. Human Ctr1 can bind two Cu(I) ions through S-atoms in a three-coordinate geometry in distinct binding sites [17]. Mutations in one of the putative binding sites (either N-terminal Met motif (MPM), or TM2 MxxxM or C-terminal Cys/His, Fig. 1) result in changes in the coordination structure of both copper ions, consistent with the hypothesis that each Cu(I) ion coordinates sulphur donor atoms from different sites [17].

The observations available up to now suggest a model for the function of the Ctr protein family in which Cu(I) first coordinates to the methionine-rich (Mets) motifs in the Ctr extracellular N-terminus. Copper is then involved in a chain of copper exchange reactions between defined Cu(I)-binding sites, involving well-defined conformational changes [17, 19, 21]. This way copper ions move through Ctr from N-terminal extracellular binding sites, furnished by the Met conserved residues, to Cys/His conserved residues in the cytoplasmic C terminus, which in turn serve as intracellular donors for Cu(I) and its mobilization to the copper chaperones. Although it is currently unclear whether the copper chaperones directly dock to the Ctr1 homotrimer to engage in cargo transfer or whether there are other intermediates in the delivery system, one study indicated that Cu(I) bound to an isolated Ctr1 C-terminal domain can be rapidly transferred to the Atx1 copper chaperone in vitro [22]. How the trimerization might also influence potential direct interactions with copper chaperones within cells or with metalloreductases on the cell surface is not yet clear.

In contrast to eukaryotes, bacteria may not have a general requirement for cytoplasmic copper [23–25]. Copper proteins are localized to the cytoplasmic membrane or the periplasm, and copper loading of these proteins could take place in these compartments. For example, *E. coli* has copper-requiring proteins only in the periplasm [26] and embedded in the plasma membrane, and similarly no cytoplasmic protein requiring copper to function is known in gram-positive bacteria [25]. Thus, many bacteria do not appear to have a requirement for intracellular copper, and the copper homeostatic machinery in these organisms may have the sole purpose of eliminating adventitious copper ions [25]. This concept is supported by the complete absence of copper chaperones in many bacteria [27], whereas in eukaryotes, copper chaperones are essential for delivering copper to enzymes such as cytochrome *c* oxidase and superoxide dismutase [5]. Nevertheless, specific copper importers that are expressed under copper-limiting conditions or in metal-sensitive *E. coli* strains have been described in the gram-positive *E. hirae* [28] and *B. subtilis* [29] and in the gram-negative *Pseudomonas aeruginosa* Q9I147 [30], but the functional meaning of the copper import process in the cytoplasm remains to be tested.

Cyanobacteria (e.g., *Synechocystis*) is the only bacterial group that has a known demand for cytoplasmic copper for the synthesis of copper-containing plastocyanin and cytochrome *c* oxidase [31]. Many cyanobacteria contain internal membrane-bound compartments called thylakoids, which are discrete from the periplasm. Thylakoids are the site of both the photosynthetic and respiratory electron transport processes involving the copper protein plastocyanin and, though unusual for a bacterium, cytochrome *c* oxidase. Therefore, cyanobacterial thylakoids are currently the only location for copper enzymes known to require that copper enters a bacterial cytoplasm. Copper is trafficked to cyanobacterial thylakoids through the actions of two P-type ATPases, CtaA and PacS [31, 32]. A metallochaperone (Atx1) interacts with the N-terminal domains of the two ATPases and is presumed to acquire copper from CtaA and donate it to PacS [33], although only this last transfer has been verified in vitro [34]. The solution structure of the cyanobacterial metallochaperone was solved by NMR and the copper site investigated by EXAFS and NMR techniques [35, 36]. The protein shares the same fold as other related proteins studied in other organisms [37–40], but, at variance with them, which have only two Cys copper ligands located in loop 1, in *Synechocystis* the copper coordination is further stabilized by a third ligand, i.e., the Nε of the His61-imidazole ring [35]. The extra histidine ligand located in loop 5 may be significant for reversing the direction of copper transfer from acquisition to donation upon interaction with the two different ATPases, PacS and CtaA. NMR and docking studies investigating the interaction between the metallochaperone and the N-terminal domain of PacS showed in fact that loop 5 was displaced from the metallochaperone metal-binding site in the interaction with the PacS partner with the removal of the His ligand from the coordination sphere, thus assisting copper transfer from the chaperone to the ATPase [34]. This transfer mechanism allows the metallochaperone to deliver copper to one ATPase (PacS) and acquire it from another (CtaA). One hypothesis may well be that His61 is not displaced from the ligand sphere during interaction with CtaA so that copper is loaded onto Atx1 from this

ATPase [34]. This mechanism has not yet been tested, however. Overall, a probable sequence of copper transfer processes between protein partners has been proposed: copper is passed from the cyanobacterial plasma membrane to the thylakoid and hence to plastocyanin and cytochrome oxidase. The important feature of this scheme is that copper reaches its destination without release into the cytosol, and this has pivotal significance in relation to metal selectivity. Some of the most recent findings show that specific residues control the specificity of complex formation between Atx1 and PacS with respect to zinc(II)-ATPase ZiaA, whose complex with Atx1 was not observed [41]. These specific protein-protein contacts favor the interaction of Atx1 with PacS rather than with ZiaA, indeed overriding the inherent higher affinity of ZiaA for copper ion versus zinc [42]. These results further suggest that the copper pathways regulating copper homeostasis are independent from those regulating zinc homeostasis. It is likely that cells have developed several mechanisms that avoid cross-intersection of the zinc and copper homeostatic pathways. Accordingly, it has been recently reported that metal availability in the cellular compartments, where the metallo-protein folds and binds the metal ion, overrides the protein metal-binding preferences, and it therefore determines the metal specificity [43].

A route for copper toward Golgi organelle

Several copper-dependent enzymes such as tyrosinase, peptidyl-amonooxygenase, and ceruloplasmin are processed in the Golgi apparatus before being inserted in cellular membranes or secreted from cells. Copper is therefore required in the Golgi apparatus. Synchrotron X-ray fluorescence microscopy has shown that a kinetically labile copper pool is localized in the Golgi apparatus [44], and in humans, the copper transfer from cytosol in the trans-Golgi network (TGN) for the formation of the holoenzymes implicates the cytoplasmatic copper chaperone Hah1 [45] and two highly homologous transporters named ATP7A and ATP7B [46, 47]. The Hah1 chaperone, which is present in the cytoplasm and nucleus of cells [45, 48, 49], binds one copper(I) ion and delivers it to ATP7A and ATP7B, which in turn are able to pump the copper ions in the Golgi organelle for subsequent incorporation into copper enzymes. In addition to this function, ATP7A and ATP7B tightly regulate the balance between copper absorption and excretion in humans. In response to rising intracellular copper levels, the two copper-transporting ATPases can traffic from the TGN membrane close to or into the plasma membrane or to large endosomal-like vesicles [50]. The copper(I) ions are then removed from cells by pumping directly across the plasma membrane or by exocytosis, i.e., the copper(I) ions are pumped from the cytosol into vesicles that are subsequently released into the extracellular environment through fusion of the vesicles themselves with the cell membrane [51, 52]. This trafficking route of both ATPases constitutes the molecular basis of the regulation of copper absorption and excretion in the human body. Ingested copper is indeed transported by ATP7A through the basolateral membrane of enterocytes into the portal blood (and hence to other tissues), determining copper absorption in the human body [53]. Conversely, ATP7B is responsible for biliary secretion/excretion of copper in hepatocytes with the redistribution of ATP7B to a cytoplasmic vesicular compartment localized in proximity to the hepatocyte membrane to eliminate copper [52]. The regulation of copper concentration in human cells can however differentiate, depending on the human tissue involved. For example, ATP7A or ATP7B can compensate each other at least partially in the cerebellum [54], while in tissues such as the intestines, brain, or kidney, the disease-induced inactivation of ATP7A is not compensated for by ATP7B even when coexpressed in the same cells [55]. These observations suggest specific, not completely overlapping, functional roles for the two copper(I)-transporting ATPases and/or distinct mechanisms of regulation. Mutations or deletions in the gene encoding the ATP7A protein are associated with a lethal childhood disorder named Menkes's disease (MD) [46, 56], while mutations or deletions in the gene encoding ATP7B protein result in an autosomal recessive disorder of copper metabolism named Wilson's disease (WD), which leads to liver disease and neurological disorders [47, 57]. In MD, the non-functional ATP7A impairs copper export from the enterocytes, resulting in copper accumulation in intestinal cells and decreased copper transport to the blood and other tissues. In WD, affected individuals exhibit excessive copper accumulation in the liver, deficient holoceruloplasmin biosynthesis and a marked impairment in biliary copper excretion.

ATP7A and ATP7B belong to the family of the so-called P-type ATPases, which catalyze a self-phosphorylation reaction (autophosphorylation)—a unique feature of this family. The latter also includes the well-characterized Ca^{2+}-ATPases, Na^{+}/K^{+}-ATPases and H^{+}/K^{+}-ATPases, among others [58]. All P-type ATPases are transmembrane ion pumps that use the energy arising from ATP hydrolysis to transport ions across cell membranes. They are multidomain membrane proteins that exhibit similarities as well as distinct differences in architecture [59]. The basic core architecture comprises a hydrophilic region protruding into the cytosol, which contains the Actuator domain (A-domain) and the ATP-binding domain, which in turn can be further separated into two smaller domains named Phosphorylation domain (P-domain) and Nucleotide-binding

domain (N-domain). Another region common to all P-ATPases consists of a number of transmembrane helices involved in the formation of an intramembrane channel. These regions constitute the catalytic core on these ion pumps sharing a common catalytic mechanism, which was originally proposed for P_{II}-ATPases (Na^+,K^+-, Ca^{2+}- and H^+,K^+-transporting ATPases) and whose key feature is the formation of a transient acyl-phosphate intermediate upon ATP hydrolysis. ATP binding to the N-domain is the first step, together with the metal ion binding to the TM region; the enzyme is subsequently phosphorylated at an invariant aspartate residue in the highly conserved DKTG sequence located in the P-domain (Asp1044 in ATP7A and Asp1027 in ATP7B) with concomitant metal release. The intermediate is then dephosphorylated by the invariant TGE sequence of the Actuator domain. This catalytic cycle follows the classical E1/E2 scheme (Post-Albers) [60, 61] which postulates that in the E1 state, the binding sites within the TM region have high affinity for the metal ions and are exposed to the cytoplasm, whereas in the E2 state, the binding sites have low affinity and face the extracellular compartment (vesicular/luminal), with consequent metal release.

Based on the P-type ATPases structural organization, and in particular on the number and position of transmembrane segments, these ion pumps have been separated in five groups, which are referred to as type I–V [62]. The five major branches, proposed in 1998 by Axelsen and Palmgren, are type I ATPases (heavy metal pumps), type II ATPases (Ca^{2+}-ATPases, Na^+/K^+-ATPases and H^+/K^+-ATPases), type III ATPases (H^+ and Mg^{2+} pumps), type IV ATPases (phospholipid pumps) and type V ATPases (a group of pumps having no assigned substrate specificity). Within these groups a number of different subtypes can be distinguished [62]. Moreover, phylogenetic analyses have shown that the first group (named type I or P1) encompasses also some relatively uncommon bacterial ATPases that feature an organization in multiple protein subunits and are involved in potassium transport. Recently, a completely new classification of the P-type ATPases has been proposed, which subdivide them in nine families on the basis of the substrate specificity [63]. Copper(I)-transporting ATPases belong to the group IB [P_{IB}-(sub)type] according to the earlier classification (http://www.patbase.kvl.dk/), while they constitute family 5 within the new classification system ([63]; http://www.tcdb.org/tcdb/).

In the copper(I)-transporting ATPases, eight transmembrane helices define the ion channel, and a highly conserved CPC sequence in the sixth transmembrane helix (TMH) is one of their specific signatures (Fig. 2). Their transmembrane region is characterized by a reduced number of TMHs (eight) and their particular distribution with respect to the cytosolic loop forming the ATP-binding domain. The P_{IB}-type ATPases have indeed only two TM helices on the C-terminal end of the ATP-binding domain, while P_{II}-ATPases have six. Another distinguishing feature of copper(I)-transporting ATPases is the presence of a long N-terminal tail, which contains a variable number of 70-aa independently folded domains similar in sequence [27] and each of them containing a highly conserved metal-binding CXXC sequence (Fig. 2). The number of the N-terminal metal-binding domains (NMBDs) increases with evolution, i.e., while they are at most two in bacteria and archea; in eukaryotes systems they increase from two in yeast to four in *D. melanogaster* up to six in the majority of mammalians [27].

To date, there is no structural information for an intact P_{IB}-type ATPase in the various catalytic intermediates able to elucidate the molecular details of the catalytic cycle and the interplay of the cytoplasmatic domains. However, several X-ray and NMR studies have been successfully used to solve the structures of the three cytoplasmatic soluble domains of copper(I)-transporting ATPases, A-, P- and N-domains, from bacteria and human, both as isolated domains (i.e., A-domain [64, 65] and N-domain [66, 67]), and as composite N- and P-domains [68–70]; also NMR studies have provided structures of various isolated or double-domain constructs of NMBDs in different metallation states [71–75].

The solution structure of A-domain is characterized by seven antiparallel β-strands arranged in two sheets packed against each other and forming a distorted double-stranded beta-helix fold (Fig. 2). Two additional β-strands form a β-hairpin structure flanking the two sheets. Finally, two helices, which are the cytosolic extension of the fourth and fifth transmembrane helices (TMHs) of the enzyme, are present at the N- and C-termini of the structure. The A-domain structures of ATP7A and ATP7B, recently solved by NMR [65], showed that the TGE motif, which is required for the dephosphorylation step of the catalytic cycle, was properly oriented for the interaction with the P-domain (Fig. 2). Notably, when comparing the structures of the related SERCA Ca^{2+}-transporting P-type ATPase solved in states that represent the various intermediates along the catalytic cycle [76, 77], it appears that this loop undergoes only minor structural rearrangements for the protein backbone, essentially limited to the Glu residue. The observed rigidity of the TGE loop in the A-domain of ATP7A [65] suggests that this behavior may be common also to copper(I)-transporting ATPases. Moreover, in the mammalian SERCA, the interaction between the loop containing the TGE motif and the P-domain appears to be critical as it drives the rotation of the A-domain with a subsequent rearrangement of the transmembrane helices, leading to metal release from the transmembrane site. Although the different disposition of the TMHs in the

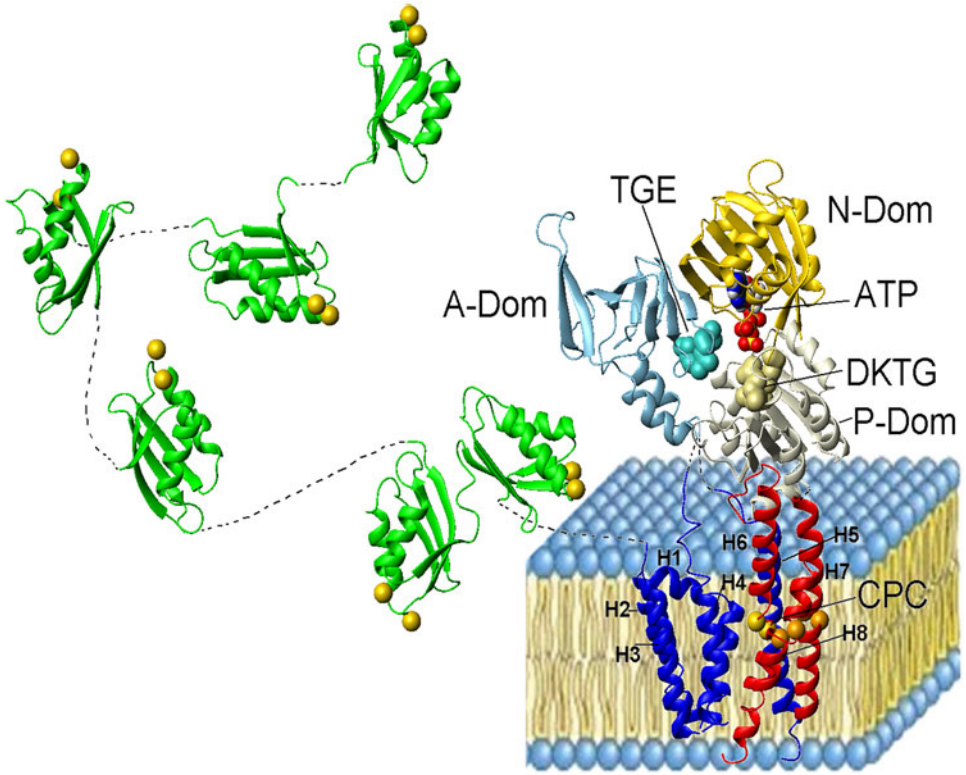

Fig. 2 Schematic representation of the topology of copper(I)-transporting ATP7A and ATP7B in analogy with SERCA. The topological scheme includes the A-domain (PDB 2KIJ), the ATP-bound N-domain (PDB code 2KMV) and each NMBDs (domain1 PDB code 1KVJ; domain2 PDB code 1S6U; domains 3–4 PDB code 2ROP; and domains 5–6 PDB code 2EW9) structures. The structure of the P-domain was modeled using as template the structure of corresponding domain in the homologous protein from *A. fulgidus* (PDB code 2B8E). The structural arrangement of the eight transmembrane helices is obtained from the cryoelectron microscopy model of CopA from *A. fulgidus* (PDB code 2VOY). The copper(I)-binding cysteines in both N-terminal domains and transmembrane CPC region are shown as *yellow spheres*. The predicted position of the residues constituting site II in the transmembrane region are shown as *orange spheres*

copper(I)-transporting ATPases might require different TM rearrangements, the structural and dynamic similarities suggest an equivalent mechanism between SERCA and copper(I)-transporting ATPases for metal release from the TM gate.

The solution structures of human A-domain also allowed us to investigate the possible mechanisms by which missense mutations in both ATP7A and ATP7B can adversely affect the enzyme activity eventually leading to, respectively, MD and WD [65]. The mutations can be broadly divided in two groups, one including residues that are buried within the protein interior, the other including residues located in regions possibly involved in interdomain interactions. The first group of mutations can influence the function of the enzyme by altering the fold of the domain or by impairing the network of communication between its different regions, and particularly between the TGE motif and the helices. The second group of mutations can impact the phosphatase activity of the A-domain by altering its interaction with the other cytosolic domains of the enzyme [65].

The structure of the P-domain within the entire copper-transporting ATPase ATP-binding domain located between the sixth and the seventh TMHs consists of a sheet formed by six parallel β-strands sandwiched between six α-helices, three on each side of the sheet ($\alpha\beta\alpha$ Rossmann fold, Fig. 2) [68–70]. The structures of the entire bacterial ATP-binding domain and those of the human isolated N-domain reveal that the latter consists of a twisted six-stranded, antiparallel β-sheet, flanked by two pairs of α-helices, one on the concave side of the sheet and the other on the convex one. In the entire ATP-binding the P- and N-domains are joined by two short loops, which define the so-called hinge region. The latter allow the two domains to undergo structural rearrangements simultaneous to nucleotide binding and the transient phosphorylated intermediate formation. Several highly conserved residues among all P-type ATPases are present within the hinge region and form a hydrogen bond network, which can be important for stabilizing the conformation of the loops located in the P-domain and containing the conserved DKTGT and TGD motifs. The latter are critical for enzyme phosphorylation during catalysis.

The structure of the P-domain, as well as that of the A-domain, are very similar to those of other P-type ATPases such as the mammalian SERCA Ca^{2+}-transporting ATPase (PDB codes 1IWO, 1SU4, 1VFP [58, 76]), the mammalian Na^+,K^+-ATPase (PDB code 1MO8 [78]) and the bacterial Kdp-ATPase (PDB code 2A00 [79]), in agreement with the fact that their sequences are overall the most conserved within all P-type ATPases [80]. In particular also the conserved phosphorylation loop in P-domain (DKTGT), which contains the invariant Asp residue, adopts similar conformation in the copper-transporting ATPases and in several SERCA structures [58, 76, 77]. Despite their low sequence similarity, even the core architecture of the N-domain of copper-transporting ATPases is shared by the corresponding domains of other P-type ATPases. In copper-transporting ATPases the ATP-protein interactions involve the same regions as found in other well-characterized P-type ATPases such as SERCA and Na^+/K^+-ATPases. A common feature of all structurally characterized N-domains is the presence of a hydrogen bond between a carboxylate group (that of Glu1081 in the case of ATP7A and Glu1064 in ATP7B) and the amino group of the adenine ring. However, there are notable variations in the way each of N-domains binds ATP. Copper(I)-transporting ATPases feature indeed a network of hydrophobic interactions [67, 70] instead of the commonly observed π–π stacking interaction with an aromatic side chain, present in the other P-type ATPases (Phe487 in the N-domain of SERCA [58] and Phe475 in the Na^+,K^+-ATPase [78, 79]. Moreover, at the rim of the ATP-binding cavity, there are positively charged amino acids (Arg489 and Arg506 in Ca^{2+}-transporting ATPase [58]), whose side chains interact with the phosphate groups. While in the copper(I)-transporting ATPases the adenine ring of ATP is located in a hydrophobic cavity formed by His1086, Pro1087, Leu1088, Gly1089, Ile1119, Ile1182, Val1214 and Ile1228 (ATP7A numbering) and any positively charged residue give rise to π-cation interactions with ATP. In ATP-binding domain of the human copper(I)-transporting ATPases, Arg506 is substituted by an histidine residue (which is His1086 and His1069 in ATP7A and ATP7B numbering, respectively). Mutation of this His to Gln in the ATP7B protein causes WD. This residue is responsible for orientating the γ-phosphate of ATP toward the Asp to be phosphorylated, making hydrogen bonds with the α- and β-phosphates and probably stabilizing the transient phosphorylated intermediate [70].

Although the structure of a construct containing all six domains of the NMBDs region (ca. 630 amino acids long) of ATP7A or ATP7B has yet to be determined, solution structures of each copper-binding domain showed that they possess a conserved $\beta\alpha\beta\beta\alpha\beta$ ferredoxin fold and bind one copper(I) ion with similar affinities by the two Cys residues of a CXXC motif (Fig. 2). NMR characterization of the N-terminal copper-binding cytosolic tails of ATP7A and ATP7B produced as full-length or in two/three domain constructs showed that the single domains 1–4 reorients in solution more freely than the domains 5–6, which are indeed bridged by the shortest interdomain linker [81–85] (Fig. 2). All the domains are able to receive copper(I) from the cytoplasmatic metallochaperone copper(I)-Hah1. The latter is an ubiquitously expressed protein of about 70 amino acids (68 in humans), which contains a highly conserved copper-binding site, CXXC, through which it is able to bind one copper(I) ion with an affinity slightly lower than the majority of the NMBDs [86]. The structures of apo and Cu(I)-loaded Hah1 are available in solution [38], while in the solid state Hah1 structure is available in complex with different metal ions: copper(I), cadmium(II) and mercury(II) [87]. Hah1 adopts, as each of the NMBDs of ATP7A and ATP7B, a $\beta\alpha\beta\beta\alpha\beta$ ferredoxin fold regardless of the presence of the metal ion. The solution structure of monomeric copper(I)-Hah1 is quite similar to the crystal structure of the same protein, but they differ in the metal coordination. In the crystal two protein molecules of a dimeric Hah1 were found to coordinate a single metal ion, while in solution the protein is monomeric with the copper ion coordinated only by two cysteine residues of the same molecule with a sulfur–copper-sulfur angle of $160° \pm 25°$ [38]. This value is consistent with the linear two-coordinate coordination observed through EXAFS data [88]. Conformational rearrangements upon copper(I) binding are small, the most prominent feature being a movement of the side chain of Lys60 towards the copper(I) site in the holo-protein with respect to the apo form. This Lys60 rearrangement upon metal binding could be due to the variation in electrostatic charge of the metal-binding loop, where the two metal-binding cysteines deprotonate to bind the mono-positive copper(I) ion, with a net total charge variation of -1. The closer position of Lys60 to the copper site can therefore contribute to stabilizing the copper adduct. This Lys has been also shown to play an essential role for copper transfer to NMBDs [87]. At variance with the metallochaperone, in the NMBDs a Phe or Tyr residue is present in the corresponding Lys position (C-terminus of helix α2). This residue is part of a network of hydrophobic contacts involving also the conserved Met, located two residues before the metal-binding loop, the second metal-binding Cys and a conserved Leu in the loop connecting strands β2 and β3 [72, 74]. Still maintaining this hydrophobic core close to the copper(I)-binding site, the third domain [89] of the cytoplasmatic tail presents some peculiarities with respect to the other five domains. Instead of a Phe/Tyr residue, there is a Pro that makes helix α2 shorter, while a Tyr, located three residues further in the sequence from the Pro, plays the role of the missing Phe. In

conclusion, the structural and metal-binding features of the Hah1 chaperone and of NMBDs are very similar, but specific differences determine their functional properties.

Recent data revealed that Hah1 may have a second function in addition to that of metallochaperone, as it can represent a novel mammalian copper-dependent transcription factor regulating cell proliferation [48]. Until now, only one gene, in mammalian genomes, was identified to encode a copper-responsive transcription factor named MTF1 (metal transcription factor 1), which regulates the metal-dependent expression of metallothioneins. Many transcription factors bind to DNA repeats as dimers to regulate transcription of the target genes. Previous evidences of copper-responsive transcription regulators that bind to DNA repeats existed in bacteria [90], some of which are forming copper-dependent homodimers. X-ray crystallography and biochemical analyses indicated the possibility of a copper-dependent Hah1 homodimerization both in vivo and in vitro [87, 91], which would initiate transcription in the nucleus. These data indicate that Hah1 can exploit a dual function as a copper chaperone and as a transcription regulator, even if how this protein can discriminate between these two functions is not yet understood. In contrast to the nuclear distribution of Hah1, the yeast homologue Atx1 is located only in the cytoplasm. Whereas copper has been shown to play a significant role in the regulation of gene expression in yeast, the same has not been yet demonstrated in mammalian cells [92]. It is therefore possible that the observed differences in nuclear localization of these copper chaperones may reflect fundamental differences in the mechanisms of copper homeostasis in these organisms.

The thermodynamic gradient for metal transfer between Hah1 and the NMBDs is low, but the metal transfer is rapid, particularly thanks to the high solvent-exposure of their binding sites and to specific interactions between the partners [81, 93]. However, copper(I)-Hah1 does not interact equally with each domain at the Hah1:ATP7A/B ratio range found in vivo, but it forms copper(I)-bridged intermolecular adducts with domains 1 and 4 of ATP7A or 1, 2 and 4 of ATP7B. However, copper(I) transfer from Hah1 to the other domains still occurs without accumulation of a detectable amount of a protein-protein adduct [81, 83]. Metallation of these last domains does not require the formation of copper(I)-mediated adducts with other metal-binding domains within the cytosolic tail, as shown by mutagenesis data for both ATP7A and ATP7B [81, 83]. In particular, domain 6, which is the closest to the transmembrane domain of the ATPases and is known to increase the catalytic activity of the pump [94, 95], can directly receive copper from copper(I)-Hah1. However, a mechanism in which copper(I)-Hah1 first forms an adduct with domains 1, 2 or 4, and then the copper(I) ion is transferred by one of these domains to domain 6 or 5, can also contribute [85]. Copper is required for the formation of a weak, reversible interaction between the partners [81, 83], indicating that the intermolecular adducts are metal-mediated, analogously to the homologous yeast Atx1/Ccc2a system [96]. It also appears that electrostatics is important in selecting which domain of the ATPase is able to form detectable amounts of the metal-mediated adduct with Hah1 [97]. Moreover, Lys60 of Hah1 has been shown to play an essential role in the metal-mediated adduct formation [98].

The copper-translocation site (CPC motif) in the sixth TM helix of ATP7A and ATP7B can be targeted directly by copper(I)-Hah1 and/or by the NMBDs of the ATPases, the last one once metallated by copper(I)-Hah1. Mutations of the two cysteines of CPC motif impair enzyme function [99, 100]. These mutants are unable to bind copper, but still are able to bind ATP [100]. Recently, it has been shown that in the *Archaeoglobus fulgidus* copper-transporting ATPase CopA, together with the CPC motif, other invariant TM amino acids form two copper(I)-binding sites with trigonal coordination [101]. Site I is constituted by the two Cys of the CPC motif and a Tyr in the seventh TM helix, while an Asn in the seventh TM helix and a Met and Ser in the eighth TM helix form the site II. These residues are the only fully conserved amino acids in the transmembrane region of the copper(I)-transporting ATPases. Both TM-binding sites bind copper(I) with an affinity ($Ka^1 = 1.12 \pm 0.25\ fM^{-1}$ and $Ka^2 = 1.30 \pm 0.22\ fM^{-1}$) higher than those of Hah1 and of NMBDs, thus preventing its backward release to cytoplasm [101]. Even if it can be proposed on the basis of copper(I)-dependent phosphorylation [102] and yeast functional complementation studies on ATP7B protein [103] that a thermodynamically shallow gradient allows for copper(I) routing from the chaperone to NMBDs and from these to the TM-binding sites, the transfer of copper(I) from a NMBDs to the TM-binding sites has not been demonstrated. Moreover, since in bacterial ATPases removal of NMBDs or mutation of their metal-binding Cys residues does not result in loss of copper transport ability, the model where copper(I)-Hah1 in humans can transfer the copper ion directly to the TM-sites can be considered a plausible alternative. However, one should also consider the possibility that there may be a number of models of delivery of copper to the ion channel, one involving the NMBDs and another independent of NMBDs, and the various assays, used to investigate the functional role of the NMBDs in the copper transport activity, can measure these distinct modes of copper transfer. For example, according to the first model it has indeed been observed that domain 6 only is required for normal copper transport activity of ATP7B, even if it is not essential for it [103]. Therefore, the proposed model was

that the copper-binding motifs closest to the transmembrane channel of ATP7B are directly involved in copper transport, transferring copper to residues within the channel, for subsequent translocation across the membrane, while the remaining N-terminal motifs were not directly involved in copper transport to the transmembrane sites [95]. On the contrary, experiments on the well characterized CopA from *A. fulgidus* support the NMBDs independent model, showing that the copper chaperone CopZ, homologous of the human Hah1, is able to transfer a copper(I) ion to one of the TM-metal-binding sites [104, 105]. It has been also shown that ATP-binding somehow regulates the accessibility of the second transmembrane site [105]. Indeed, nucleotide binding to the ATP-binding domain is required for metal occupancy of the second transport site, while the first site is occupied independently on the presence of ATP [105]. As in other P-type ATPases, metal binding to transmembrane metal-binding sites is required for enzyme phosphorylation and subsequent copper transport [105, 106]. The copper(I) binding within the TM sites occurs however in CopA without the participation of the other copper(I)-binding cytosolic domains present in the ATPase, suggesting that they are not required for ATPase activation by chaperone [105]. This finding is in agreement with the fact that for a small number of bacteria, metallochaperones were detected in the same operon of a P-type ATPase lacking any NMBDs, suggesting that the ATPase does not strictly need to receive its metal substrate through the cytosolic NMBD first [107]. The functional role of the NMBDs in copper transport has therefore still not been fully clarified and remains somewhat controversial. On the other hand, the removal of NMBD in *A. fulgidus* CopA yielded enzymes with higher enzyme turnover, suggesting a regulatory/self-inhibitory role for the NMBDs [104]. On the basis of cryoelectron microscopy, papain digestion and cross-linking studies on CopA [108–110] and structural characterization of ATP7A/B A-domains [65], and taking into account the critical role of this domain in SERCA in regulating the metal affinity within the transmembrane sites acting as the actuator of the transmembranes gating mechanism [58, 77, 111], it has indeed been suggested that domains 5 and/or 6 of the NMBDs may interact with the cytoplasmatic region (A- and/or ATP-binding domains, Fig. 2), contributing to regulating the enzyme turnover. A similar role is played by an additional (with respect to P_{1B}-type ATPases) N-terminal sub-domain (dubbed A1) in the actuator domain of SERCA. The latter is constituted by an A1 sub-domain, anchored through hydrophobic interactions to A2 sub-domain, which resembles the A-domain of P_{1B}-type ATPases. In SERCA both domains play a critical role in transmitting to the membrane region the relative movement of the soluble catalytic domains, directly affecting the orientation of the transmembrane helices [76, 77]. The hydrophobic region, involved in the A1–A2 domain recognition, is also conserved on the surface of the A-domain of P_{1B}-type ATPases [65] and might be instrumental for the communication with the NMBDs, thus forming a unit that could be functionally equivalent to the entire A-domain of SERCA. Copper binding to NMBDs could regulate such communication. Stokes and collaborators [108] proposed indeed that in the absence of copper(I) ion the NMBD of CopA would restrict A-domain and N-domain movement playing an autoinhibitory role. Upon copper binding, the NMBD would be displaced and thus allow the motions of the catalytic domains essential for the enzyme turnover.

The above studies suggest that the first four N-terminal NMBDs, which are only present in more highly evolved eukaryotic organisms, are not necessary and are not directly involved in copper transport. This raises an interesting question: what is their functional role?

At variance with less evolved organisms, the ATP7A and ATP7B proteins can traffic from the TGN to the plasma membrane or intracellular vesicles in response to copper concentration. In particular, the localization of ATP7A or ATP7B is dependent upon the balance between the rate of formation of the transient acyl-phosphate intermediate, which stimulates trafficking to the plasma membrane or vesicular compartments, and the rate of protein dephosphorylation, which determines back-trafficking from the membrane to the TGN [112]. For both ATP7A and ATP7B, formation of this acyl-phosphate intermediate was dependent on copper, and the copper chaperone Hah1 has been found to stimulate the acyl-phosphate formation [113]. The phosphorylation process indeed requires the presence of copper(I) ion bound to the ATPase, and Hah1 can directly control the amount of copper bound to it, being able to transfer or remove copper ions from its metal-binding sites. In particular, since formation of the acyl-phosphate intermediate is not induced by apo-Hah1, the presence of copper and not just of the metallochaperone is essential for ATPase activity. Formation of the acyl-phosphate intermediate is also partly affected by the cytosolic NMBDs and by the intramembrane copper(I)-binding site (CPC), whose presence enhances the efficiency of the trafficking process [114]. Since domains 1–4 are targeted by Hah1 and an interaction between the ATP-binding domain of ATP7B and its cytosolic tail has been observed, regulating the affinity for ATP of the first [115], we can suggest that the formation of the copper(I)-bridged adduct of Hah1 with domains 1, or 2 or 4, or a combination of them, is important in determining the balance between forward and back-trafficking. At high copper levels, the adduct of Hah1 with one or more domains 1, 2 and 4 of ATP7A or ATP7B accumulates in the cell. The adduct could contribute to enhancing the rate

of phosphorylation of ATP7A and ATP7B or to stabilizing the acyl-phosphate intermediate with respect to dephosphorylation, or both. By increasing the life time of phosphorylated ATP7A and ATP7B, the copper(I)-dependent complex between Hah1 and domains 1 or 4 would thus promote the localization of ATP7A and ATP7B to the plasma membrane or vesicular compartments, leading to copper(I) export out of the cytosolic space. In conclusion, the first four N-terminal NMBDs may act to induce conformational changes in the ATPases, sensing cytosolic copper concentrations, thereby regulating the redistribution rate of copper(I)-transporting ATPases within the cell.

Recently, phosphorylation sites within the N-terminal cytosolic tail of both ATP7A [116] and ATP7B [117] have been identified. Interestingly, for both ATPases, a copper-dependent hyperphosphorylation process, which necessitates the presence of the N-terminal region, has been observed. The copper-dependent hyperphosphorylation is predicted to regulate catalytic activity and trafficking responses. Accordingly, it has been recently shown that yeast copper(I)-transporting ATPase activity is regulated by Ser258 phosphorylation [118] and that the phosphorylation process of specific serine residues in ATP7A regulates its sub-cellular localization [116]. A proposed model suggests that the re-localization of copper(I)-transporting ATPases from the TGN to vesicles is associated with a hyper-phosphorylated state of copper(I)-transporting ATPases, while recycling back to the TGN with dephosphorylation to the basal level [119]. It is possible that the phosphorylation sites become exposed as a consequence of copper-induced conformational changes, which include the interaction between the NMBDs and the other cytoplasmatic domains or with the metallochaperone Hah1. It has recently been observed that the re-localization of ATP7A can also occur upon activation of the synaptic glutamate receptor (the NMDA receptor) in hippocampal neurons, resulting in an associated efflux of copper that is dependent on ATPase function [120]. Although it has repeatedly been shown that the addition of copper to cell culture medium results in the trafficking of ATP7A and ATP7B, NMDA receptor activation-dependent trafficking of ATP7A is independent of copper concentration [120]. Since the induction of kinase-mediated phosphorylation events at the N- and C-termini of ATP7A has been observed [116] [although the identity of the kinase(s) acting on ATP7A remains unknown], it could be speculated that changes in intracellular calcium concentration induced by the opening of the NMDA receptors [120] result in ATP7A proteins that are hyperphorylated by such calcium-dependent protein kinase(s). The underlying mechanisms linking copper homeostasis to the receptor responsive trafficking of copper(I)-transporting ATPases are not currently understood.

A cytoplasmic copper pathway: the metallation of Cu,ZnSOD1

One of the cellular defense systems from oxidative insults is the antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1). SOD1 is a copper-zinc enzyme and catalyzes the disproportionation of superoxide anion to oxygen and hydrogen peroxide [121]. In eukaryotes, it is mainly localized in cytosol with a smaller fraction in the mitochondrial intermembrane space (IMS) [122, 123]. It has also been reported in nuclei, lysosomes and peroxisomes [124]. Both the primary sequence and the three-dimensional structure of SOD1 are highly conserved from prokaryotes to eukaryotes [125]. SOD1 forms a tight non-covalent dimer with a dissociation constant of $\sim 1.0 \times 10^{-10}$ M^{-1} for the human enzyme [126], and each subunit has an immunoglobulin-like β-sandwich fold with an intrasubunit disulfide bond (Fig. 3). A copper and a zinc ion are embedded in each subunit; the Cu(II) ion is coordinated by four histidines (His46, 48, 63 and 120), forming a distorted square plane, and interacts with a water molecule. One of the histidines, His 63, is also a ligand of the Zn(II) ion, which completes its coordination sphere with three other amino acid residues (His71, His80 and Asp83) in a distorted tetrahedral arrangement. Binding of the Zn(II) ion is not essential for the dismutation reaction, but confers high protein stability to SOD1 [127]. An intramolecular disulfide bond is highly conserved in the SOD1 proteins from all species found so far; it confers stability to the protein fold and plays a structural role through the formation of a hydrogen bond with Arg 143, by which the side chain of Arg 143 is optimally oriented for uptake of the superoxide anion [128].

While copper and zinc incorporation into the apo-SOD1 polypeptide can be achieved in vitro by addition of the corresponding metal salts [129], the process is more complex in the cell. The copper chaperone protein for SOD1, CCS, was first identified in the yeast strain *Saccharomyces cerevisiae* through a genetic approach [130] and then emerged as an important posttranslational regulator of SOD1 structure, function and physiology [131]. Yeast cells lacking the CCS gene exhibit the same phenotypes as sod1Δ null mutant and do not show SOD1 activity, even if SOD1 expression remains unchanged relative to the wild type. SOD1 activation in yeast cells is thus strictly dependent upon the existence of CCS. CCS proteins have been identified in various species including humans, rodents, insects and plants. While CCS is 15- to 30-fold and 5-fold less abundant than SOD1 in mammalian [132] and yeast cells [6], respectively, the cellular distribution and expression of CCS appear to parallel those of SOD1. It has also been found that post-translational modifications of apo-SOD1 by CCS requires oxygen [133]. The exposure of

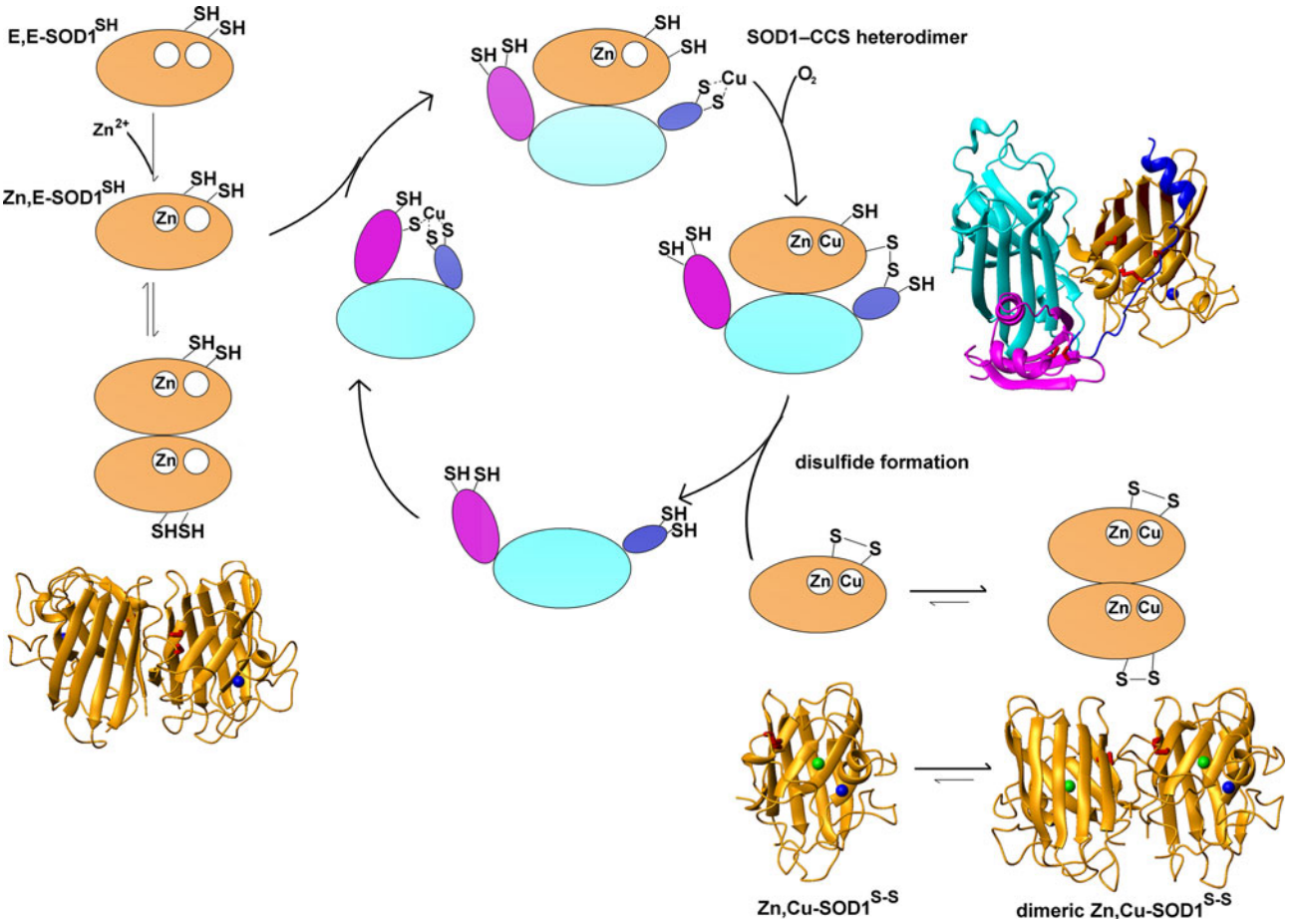

Fig. 3 Proposed mechanism of copper transfer and CCS-dependent activation of Cu,Zn-SOD1$^{S–S}$. The crystal structure of the yeast SOD1-yeast CCS heterodimeric complex ([131], PDB code 1JK9) and the structures of dimeric Zn,E-SOD^{SH}, Cu,Zn-SOD^{S-S} and monomeric Cu,Zn-SOD^{S-S} (PDB codes 2AF2, 1L3N and 1BA9, respectively) are shown. CCS domains I, II, III are in *magenta*, *cyan* and *blue*, respectively. Zn(II) and Cu(I) ions are shown as *blue* and *green spheres*, respectively. The side chains of cysteines are shown as *red sticks*

anaerobically grown yeast to air induces CCS expression and activation of SOD1 within an hour. Furthermore, in vitro experiments have shown that CCS can activate apo-SOD1 under aerobic conditions, but not in the absence of oxygen [133].

CCS is a 28-kDa protein comprised of three domains (domain I, domain II and domain III hereafter, Fig. 3). The first in the N-terminus region (~8 kDa) has a structure similar to that of copper chaperone Hah1 and contains a "CXXC" metal-binding motif that can bind one copper ion when the domain is expressed as isolated, but is not essential to SOD1 activation in cells under normal conditions [134]. A CCS mutant construct in which domain I is deleted can still activate SOD1, albeit at a much lower level. Cys to Ser mutations in the CXXC motif still exhibit 70% metallochaperone activity compared to the wild-type protein in vitro [135]. Domain II of CCS (~16 kDa) is highly homologous to SOD1, sharing 47% sequence identity and the same fold. While no metal-binding sites are present in yeast CCS domain II, all of the SOD1 zinc-binding ligands are conserved in the human protein. In fact, an equimolar amount of zinc ion is bound in human CCS domain II when overexpressed in *E. coli* [136], and zinc binding is essential to human CCS function [137], possibly because it contributes to protein stabilization. Three of the four histidine residues present in the SOD1 copper-binding site are also conserved in domain II of human CCS, with the fourth histidine replaced by an aspartate. The copper ion is not bound at this site, and SOD1 activation by CCS is still observed after mutations of these His residues to Ala [134]. Copper binding to domain II would thus not be required for copper incorporation into SOD1. Domain III of CCS is a short polypeptide (30–40 amino acids) without any tendency to form secondary structures, but is essential to CCS function [134]. It exhibits a low homology with a portion of prolyl *cis-trans* isomerase and features a CXC motif, which is highly conserved among all species. The domain III polypeptide alone is sufficient to bind a Cu(I)

ion, and the Cys to Ser mutations in the CXC motif dramatically reduce the metallochaperone activity of CCS in vivo and in vitro [134]. Copper coordination in full-length CCS is still quite controversial as, depending on the experimental conditions, variable amounts of copper ions are bound in both the yeast and human CCS proteins. Extensive EXAFS experiments have recently led to the proposal that human CCS can form two distinct clusters with different stoichiometry: a polynuclear Cu_4S_6 cluster involving extra Cys residues from domain II and a dinuclear Cu_2S_4 cluster when these extra Cys residues are unavailable [138, 139]. The functional significance of this complex copper(I) coordination in CCS is still elusive.

Given the similarity of SOD1 and CCS dimer interfaces, a monomeric CCS molecule has been proposed to interact with a monomeric SOD1 molecule through its SOD1-like domain II, thus mimicking SOD1 dimerization. In fact, when dimerization of yeast SOD1 is prevented by F50E/G51E mutations at the subunit interface, SOD1 is not activated by yeast CCS [140]. Similarly, when the corresponding amino acid residues in yeast CCS domain II are mutated (K136E/G137E), in vivo activation of yeast SOD1 is not observed [140]. These findings have supported the idea that CCS domain II interacts with the SOD1 subunit interface (Fig. 3). SOD1 is however a tight dimer, and monomeric forms of the WT holo protein have not been observed. Based on the high dimer stability of either SOD1 (K_d, 1×10^{-10} M^{-1}) [126] or CCS (K_d, 3.0×10^{-6} M^{-1} in yeast) [141], the hetero-tetramer model, in which both proteins do not need to dissociate into monomers, has been proposed [141]. However, the crystal structure of the yeast SOD1-yeast CCS complex supports the monomer-monomer interaction model (Fig. 3) [142]. An interaction between monomeric SOD1 and monomeric CCS domain II was indeed observed in the complex, with the functionally essential domain III of CCS well positioned to interact and exchange copper with SOD1. Consistent with the monomer-monomer model, it has recently been shown that the copper-bound yeast CCS can activate the disulfide-reduced apo-yeast SOD1 monomeric form [127, 133]. Indeed, a dimeric apo-form of SOD1 that already possesses the intramolecular disulfide bond does not accept copper from the chaperone and cannot participate in the hetero-tetrameric interaction between SOD1 and CCS [127]. Indeed, the SOD1 quaternary structure is dependent on the degree of posttranslational modifications and the most immature form (i.e., the disulfide reduced apo-SOD1 polypeptide) favors the monomeric state [143], which can interact with CCS through domain II.

No direct information can be obtained on the mechanism by which the copper ion is transferred from CCS to SOD1 from the structural data on the heterodimeric complex. However, an intermolecular disulfide bond is formed between Cys 57 (SOD1) and Cys 229 (CCS) (Fig. 3) in the SOD1-CCS heterodimer [144]. Since active SOD1 possesses an intramolecular disulfide bond between Cys 57 and 146, the formation of the intermolecular disulfide between SOD1 and CCS suggests the involvement of CCS in SOD1 disulfide formation. CCS thus plays a functional role not only as a copper chaperone but also as a real "chaperone" protein. Oxygen participates in the intermolecular disulfide formation as, under anaerobic conditions, the cysteine residues in SOD1 remain reduced even after incubation with copper-CCS, and the protein remains inactive [127, 133]. Oxygen itself is therefore necessary as an oxidant to form the intermolecular disulfide bond, possibly favoring the release of copper from CCS to apo-SOD1 (Fig. 3). Following copper insertion into the heterodimeric complex, copper binding in SOD1 could induce conformational changes, especially around the Cys residues, to promote the protein disulfide isomerase-like reaction from intermolecular to intramolecular SOD1 disulfide (Fig. 3). Disulfide exchange within the heterodimeric intermediate further drives dissociation of the docked complex to the individual dimeric proteins, Cu,Zn-$SOD1^{S\text{-}S}$ and apo-CCS. In this model (Fig. 3) proposed by the O'Halloran group [131], copper insertion and disulfide formation in apo-SOD1 are thus performed by CCS, but despite the important role of the Zn(II) ion in SOD1 folding and structure, little is known about how this protein acquires a Zn(II) ion in the cell. Indeed, no zinc chaperone has been identified up to now. Given that human CCS can bind a Zn(II) ion in domain II and possibly in the Hah1-like domain I, it has been proposed that CCS may also function as a "Zn chaperone" protein, but this mechanism has yet to be proved.

Posttranslational activation of SOD1 including copper/zinc binding and disulfide formation is thus regulated in a well-concerted manner. If defects are present in the posttranslational activation pathway, the SOD1 protein itself could be toxic to the cell. In particular, SOD1 protein aggregates have been found in the motor neurons of SOD1-related familial amyotrophic lateral sclerosis (ALS) patients and transgenic mouse models, and have therefore been proposed to be related to the familial form of amyotrophic lateral sclerosis [145]. Given that posttranslational modifications generally increase protein structural stability, it is expected that the most immature form [i.e., apo- and disulfide-reduced SOD1 (E,E-$SOD1^{SH}$)] is the most susceptible to aggregation, as has indeed been shown by several studies [146, 147]. In particular, it has been shown that disulfide reduction in wild-type apo-SOD1 drops the melting temperature to 46°C, which is ~45°C lower than that of holo-SOD1 and close to body temperature (~37°C), implying that a small fraction of even wild-type protein may be unfolded when

any posttranslational modifications are absent [146]. Indeed, we have recently shown that wild-type human SOD1, when lacking both its metal ions and with Cys6 and Cys111 in a reduced state, forms large, stable, soluble protein oligomers with an average molecular mass of approximately 650 kDa under physiological conditions, i.e., 37°C, pH 7.0, and 100-μM protein concentration [148]. Oligomerization involves formation of intermolecular disulfide bonds between Cys6 and Cys111. Since SOD1 aggregates have also been observed without disulfide cross-linking [149], the cysteine residues might therefore play a role in modulating the rate of aggregation of human SOD1. ALS-associated mutations might favor the most immature state of the SOD1 protein by decreasing both disulfide stability and metal affinity. Indeed, while the intramolecular disulfide bond in wild-type SOD1 is kinetically robust against reducing agents, the disulfide bond in ALS-mutant SOD1 would not persist in the reducing environment of the cytosol [150]. SOD1 has such an extremely high copper/zinc affinity that it functions as an intracellular copper/zinc sink [151, 152], but significantly reduced affinity and/or altered binding geometry of the zinc ion is found in some mutant SOD1 proteins [153]. We have recently shown that several SOD1 mutants, most of them reported as involved in ALS onset, undergo the same general mechanism of oligomerization as found for the WT metal-free protein only when they are in the metal-free form [154]. The rates of oligomerization are different but eventually give rise to the same type of soluble oligomeric species. As in the wild type, these oligomers are formed through oxidation of the two free cysteines of SOD1 (6 and 111) and are stabilized by hydrogen bonds between β-strands, thus forming amyloid-like structures [154]. SOD1 also enters the mitochondria in a demetalated state [123], and mitochondria are loci where oxidative stress may easily occur. It has indeed been shown that aggregation of mutant SOD1 in mitochondria is relevant for inducing mitochondrial damage [155]. The soluble oligomeric species formed by the apo form of either WT SOD1 or its mutants through an oxidative process may represent the precursor toxic species, whose existence would also suggest a common mechanism for ALS and fALS [148, 154]. The proposed mechanism for SOD1 WT/mutant oligomerization is absolutely general and provides a common unique picture for the behaviors of the many SOD1 mutants, which are of different natures and distributed throughout the protein. Very recently, the structural and dynamic properties of the metal-free form of WT human SOD1 and its familial amyotrophic lateral sclerosis (fALS)-related mutants, T54R, I113T, A4 V and G93A, were characterized in solution, through NMR, and/or in the crystalline state through X-ray diffraction [156]. NMR experiments show that the protein is highly disordered in solution, and samples a large range of conformations. Also, all X-ray structures show significant structural disorder in loop regions that are instead well defined in the fully metallated structures. Interestingly, the apo state only crystallizes at low temperatures, whereas all proteins in the metallated form crystallize at any temperature, suggesting that crystallization is favored when the lower temperature reduces the range of conformations sampled by the protein; one of the most stable conformations among all of those adopted by the apo form in solution is therefore selected in the crystal. The large variability of the conformational states of apo, reduced WT and mutant SOD1 is likely a primary culprit for SOD1 aggregation processes and can be one of the determinants for the onset of ALS/fALS neurodegenerative disease.

It has also become clear that CCS is not the only way to activate SOD1. Indeed, CCS-knockout mice and CCS−/− fibroblast mouse cells have been shown to retain a certain degree of SOD1 activity, suggesting a CCS-independent pathway for SOD1 activation in mammalian cells [157, 158]. This CCS-independent activation of mammalian SOD1 was then mirrored in a yeast expression system. In all organisms that have been tested until now, maximal SOD1 activity is however obtained with CCS, suggesting that CCS may represent the preferred route of enzyme activation, while the CCS-independent pathway acts as a backup under conditions in which CCS is limited [159]. Significantly, the nematode *Caenorhabditis elegans*, which does not have a CCS homologue, uses some type of CCS-independent pathway for activation of intracellular Cu,Zn-SOD1 proteins, which is highly homologous to the mammalian ones [158]. It is indeed unlikely that *C. elegans* is the only species that has evolved completely independently of CCS. In an attempt to understand the mechanism of SOD1 maturation in the absence of CCS, the maturation patterns of Cu,Zn-SOD1 molecules in organisms that are completely CCS-dependent (*S. cerevisiae*) were compared with those that use only the CCS-independent pathway (*C. elegans* SOD1) and those that employ both pathways (human SOD1) [158]. Two striking differences were noted: (1) the intramolecular disulfide in *C. elegans* Cu,Zn-SOD1 is present in the oxidized state regardless of copper or CCS conditions and, in human SOD1, is still $\approx$50% oxidized in the absence of CCS and copper; (2) CCS-independent activation of human SOD1 has been observed under hypoxic and anoxic conditions, indicating that oxygen is not required for activation of SOD1 [160]. In conclusion, SOD1 enzyme activation is in itself an intriguing process, where organisms exhibit the optimal method for activating SOD1 to fit their complexity and lifestyle.

Copper trafficking to the mitochondrion and assembly of mitochondrial copper enzymes

The mitochondrion is a cellular organelle consisting of a continuous reticulum that makes up nearly 10% of the cell volume in respiring yeast cells. A double membrane creating two internal volumes encloses the organelle. The space between the two membranes is called the IMS and is interrupted by junction points in which the inner membrane (IM) and outer membrane (OM) are in contact. The volume enclosed within the IM is known as the matrix compartment. In cells with high respiration rates, the IM is convolutedly folded into tubular structures designated cristae that are enriched in the enzyme complexes involved in oxidative phosphorylation. The mitochondrial reticulum is highly dynamic, constantly changing size and shape through fission and fusion events. The mitochondrion contains a genome in the matrix. Eukaryotic cells contain several mitochondrial genomes organized in nucleoids, the number varying depending on organisms and growth conditions. The number of gene products encoded by the mitochondrial genome varies between species. The yeast mitochondrial genome encodes only 8 proteins of the respiratory chain in addition to 24 tRNAs and 2 rRNAs. In contrast, the human mitochondrial genome encodes 13 known polypeptides of the human electron oxidative phosphorylation pathway, 22 tRNA species and 2 rRNAs. In particular, three of the mitochondrially encoded proteins in humans and yeast are subunits of cytochrome *c* oxidase (CcO), which is the terminal respiratory complex (complex IV). These subunits contain multiple cofactors critical to catalytic competence of CcO, and their delivery and insertion into the nascent holoenzyme complex require multiple proteins. Two heme *a* moieties and three copper ions are contained within conserved domains of Cox1 (*a*, a_3, Cu_B) and Cox2 (Cu_A) [161]. The mononuclear Cu_B site in Cox1 interacts with heme a_3 to form a heterobimetallic heme a_3–Cu_B center, while the Cu_A site contained within Cox2 exists as a cysteine-bridged, binuclear, mixed covalent center [162]. The catalytic core of CcO is surrounded by nuclear-encoded structural subunits, which confer stability to the holoenzyme and provide sites for the allosteric regulation of its activity. The fully assembled holoenzyme is embedded within the IM, and is further organized into higher order structures termed supercomplexes in yeast and mammals [163].

The essential need for copper in mitochondria derives from its role in the CcO enzyme, which is required for cellular utilization of oxygen. One major function of the mitochondrion is the production of ATP through the respiratory reduction of oxygen to water and oxidation of NADH and $FADH_2$. Three respiratory complexes funnel electrons obtained from the oxidation of NADH and $FADH_2$ to CcO for reduction of oxygen. Electron transfer is coupled to proton pumping across the IM resulting in a proton gradient that is used to drive ATP synthesis by the ATP synthase complex. The respiratory complexes utilize multiple cofactors for their function but, among them, only CcO requires copper for function. Copper is also needed in the mitochondrion for the protection against oxidative stress. Indeed, cellular energy production occurring in mitochondria is obviously beneficial for the cell, but also bears the risk of producing oxygen radicals that have a high redox potential, so damaging important mitochondrial components. In mammals, more than 95% of the daily consumed oxygen is reduced to water in the respiratory chain, but 1–2% of it has been estimated to be converted to O_2^- by proteins involved in the electron transport chain of mitochondria [164]. In order to avoid superoxide accumulation, which determines serious cellular oxidative damage, eukaryotic cells exploit mitochondrial SOD enzymes both in the IMS [165], as Cu,Zn-SOD1, and in matrix, as MnSOD2 [166]. Besides CcO, Cu,Zn-SOD1 is the only other known copper metalloenzyme within mitochondrion. Approximately 1–5% of total yeast SOD1 is localized within the IMS with the remainder in the cytoplasm [165].

Since the copper-binding subunits of CcO are encoded by the mitochondrial genome, its copper metallation must occur within the organelle. Mitochondrial SOD1 is imported in the IMS in the apo-configuration [123], so its metallation must likewise occur within the IMS. The presence of two copper metalloenzymes that are metallated within the mitochondrion needs a specific copper transport pathway to this organelle for the correct functioning of CcO and SOD1 enzymes. The importance of this pathway is underscored by inherited deficiencies in humans with mutations in CcO-copper assembly proteins [167] as well as by misfolding events of immature copper-depleted forms of SOD1 [148, 168]. However, how copper is trafficked from the cytoplasm to mitochondrion is still an open question. Copper transporters translocating Cu(I) ions from the cytoplasm are indeed known, but their localization is restricted to the trans-Golgi network and internal vesicles, and are not responsible for Cu(I) translocation to the mitochondrion. Initially, several candidates (Cox17, Cox19, Cox23) for the delivery of copper to the mitochondrion were proposed on the basis of their dual localization in both the cytoplasm and mitochondrial IMS as well as on the basis of respiratory-deficient effects due to a complete lack of CcO activity [169–171]. However, subsequent studies showed that they do not have any role in copper shuttling to the mitochondria, but each protein has a distinct role within the IMS in copper trafficking [172, 173]. Later, a labile copper pool was found in mitochondria of yeast and human cells, and it was proposed to be the

source of copper used in the metalation of CcO and of SOD1 within the IMS [174]. The labile copper pool is formed by a low mass ligand complex that resides within the matrix and stably binds Cu(I) in an anionic complex (CuL), but its nature has yet to be elucidated. The suggested model predicts that Cu(I) binding to the ligand within the cytosol triggers the translocation of the anionic CuL complex to the mitochondrion [175]. The CuL complex translocation model also predicts that transport occurs both into and out of the matrix. Therefore, either two transporters or a single bidirectional transporter exist within the inner mitochondrial membrane. Once the CuL diffuses through channels in the outer mitochondrial membrane, a transporter moves it from the IMS into the matrix [176]. The labile copper pool would then result in the channeling of either Cu(I) or the CuL complex from the matrix back to the IMS for subsequent use by Cox17 and CCS (Fig. 4) in mitochondrial metalation reactions of CcO and SOD1, respectively. This very appealing model is still largely speculative and needs additional data to be definitively proved.

Once copper ions reach the IMS, complex protein machinery operates in the case of CcO to correctly insert them in the nascent CcO enzyme. This process occurs in concert with the myriad of other steps implicated in the assembly of the multimeric enzyme (i.e., assembly of the subunits translated on cytoplasmic and mitochondrial ribosomes, modification of protoheme to heme *a* and delivery and subsequent insertion of this moiety into the nascent enzyme complex, along with the insertion of zinc and magnesium ions). The large array of accessory proteins known to be essential in the copper metallation of CcO are Cox11, Cox17, Cox19, Cox23, Sco1 and Sco2 (Sco2 is not required in yeast). All these accessory proteins are nuclear-encoded and translocate in the IMS exploiting differentiated mitochondrial import mechanisms in accordance to their amino acid sequence properties. Copper insertion into both Cox1 and Cox2 subunits of CcO therefore occurs, similarly to SOD1, within the IMS compartment.

Protein-mediated copper insertion into CcO

The insertion of copper into Cox1 and Cox2 in IMS seems to mirror the copper chaperone paradigm from the cytosol. That is, copper is inserted by copper-binding chaperones and co-chaperones at specific stages of the holoenzyme assembly. Cox17 acts indeed as a copper chaperone in IMS delivering copper(I) to both Sco1 and Cox11 [177]. Cox17 retains all the typical characteristic of the cytoplasmic copper chaperones such as relatively high copper(I)-binding affinity, solvent exposed metal-binding site and the presence of a lysine residue (Lys20 in human Cox17) close to the copper-binding site, which has been proposed to have a functional role in stabilizing copper binding and modulating copper transfer (Fig. 4). Although Cu(I)Cox17 can adopt multiple oligomeric states forming polycopper clusters [178], it now appears that the active conformer consists of a single Cu(I) ion coordinated to a monomeric protein that is stabilized by two disulfide bonds formed by a twin Cx_9C motif [179–181] (Fig. 4). NMR solution structure of this form of Cox17 ($Cox17_{2S–S}$) showed the presence of a coiled coil-helix-coiled coil-helix domain stabilized by two disulfide bonds, preceded by a flexible and completely unstructured N-terminal tail [179] (Fig. 4). In human $Cox17_{2S–S}$ the copper(I) ion is coordinated by the sulfurs of two consecutive cysteines upstream of the twin Cx_9C motif, representing the first example of a Cys-Cys binding motif in copper proteins. The redox couple from the double disulfide configuration $Cox17_{2S\text{-}S}$ to the fully reduced state has a midpoint potential of −340 mV, consistent with the double disulfide bonded state being the likely species in vivo [182]. However, at variance with cytoplasmic copper chaperones as Atx1, the fold of Cox17 as well as copper(I)-binding motifs are completely unrelated to its protein partners Sco1 and Cox11, which in turn have different folds and metal ligands one from the other. These structural differences of the Cox17/Sco1/Cox11 pattern versus the Atx1/Ccc2a pattern can play an important role in modulating the metal transfer processes in different ways. Similar folds with similar metal-binding ligands in the protein partners can be indeed important in determining a reversible copper transfer mechanism, as found in the Atx1/Ccc2a interaction [93, 96, 183], whereas different fold and metal ligands of the protein partners found in the Cox17/Sco1/Cox11 interactions can somehow have a role in the quantitative and directional copper transfer directly observed in vitro in the case of Cox17/Sco1 pair [181], as well as in the recognition and binding to different partners. In the case of the Cox17/Sco1 pair, the quantitative copper transfer can be driven by the higher number of Sco1 metal-binding ligands, being indeed Cu(I) ion in Sco1 coordinated by two cysteines and one histidine (see after) [184]. Finally, at variance to the Atx1/Ccc2 interaction, the copper transfer from Cu(I)Cox17 to Sco1 occurs without quantitative accumulation of the protein-protein complex, which is indeed very transiently formed in solution and detected only by ESI–MS spectrometry [181]. Up to now, this has prevented a detailed structural characterization of the Sco1/Cox17 protein-protein complex. The only available data on this matter derive from indirect assays suggesting that the surface of human Sco1 consisting of residues close to the CXXXC metal-binding motif and to the conserved His ligand may be important for Cox17 recognition [185].

Sco1 was first proposed to be implicated in copper delivery to CcO by the observation that the respiratory

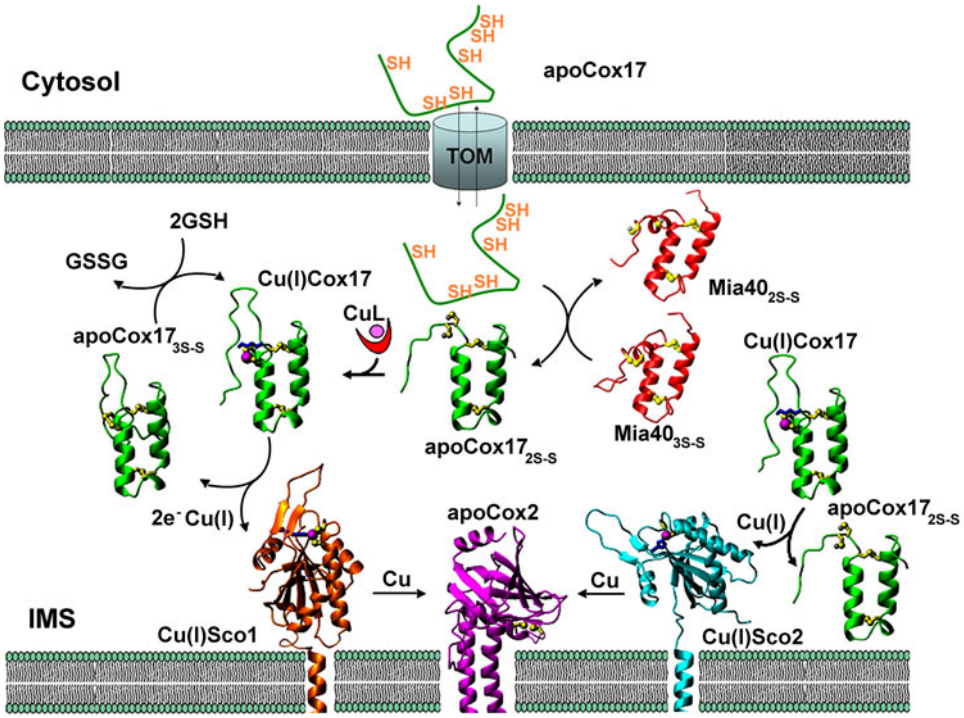

Fig. 4 Proposed mechanism of copper insertion into the Cu_A site of cytochrome *c* oxidase. The crystal structure of bovine Cox2 (PDB code 2ZXW) and the solution structures of human Mia40, Cox17, Sco1 and Sco2 (PDB codes 2K3J, 2RN9, 2RNB, 2GVP, 2GT6) in their different metal or redox states are shown. Cysteine residues involved in copper binding or disulfide bond formation are shown as *yellow sticks*. Lys 25 in Cox17 and His copper(I) ligands in Sco1 and Sco2 are shown as *blue sticks*. Copper ions are shown as *magenta spheres*. The unknown transporter (L) proposed to be involved in copper availability for apoCox17 in the IMS is shown in *red*. The TOM complex involved in Cox17 protein import into the IMS is schematically shown

deficient phenotype of a *cox17-1* yeast mutant was suppressed by overexpression of *SCO1* or a related gene designated *SCO2* [186]. Sco1 and Sco2 are highly similar proteins tethered to the IM by a single transmembrane helix at the N-terminus (Fig. 4). The function of Sco1 is dependent on its single transmembrane helix since targeting of Sco1 to IMS as a soluble protein yields a non-functional protein [187]. Cells lacking yeast Sco1 are respiratory deficient and are devoid of CcO activity, while yeast *sco2*Δ cells lack an obvious phenotype associated with respiration [186]. Cox2 protein levels are markedly reduced in yeast *sco1*Δ cells and absent in yeast *sco1*Δ, *sco2*Δ double null cells [188]. In contrast to yeast, both Sco1 and Sco2 are required in humans for cellular respiration [189]. Mutations in either gene produce a severe CcO deficiency that results in early onset diseases with a fatal clinical outcome; however, human *SCO2* patients present neonatal encephalocardiomyopathy, whereas *SCO1* patients exhibit neonatal hepatic failure [190]. 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 [189]. Studies with immortalized fibroblasts derived from *SCO1* and *SCO2* patients suggest that human Sco1 and Sco2 have non-overlapping but cooperative functions in CcO assembly [189], even if the difference in their role has not been yet fully elucidated.

Both human Sco1 and Sco2 have a C-terminal globular domain protruding into IMS (Fig. 4). Their globular domain possesses a thioredoxin fold consisting of a central four-stranded β sheet covered with flanking helices [184, 191] (Fig. 4). Both proteins pass from an open and conformationally mobile state to a closed and rigid conformation upon copper(I) binding. However, Sco2 behaves in both apo and copper(I)-bound forms as a protein with a dramatic backbone fluxionality, which is distinct with respect to the high structurally rigidity of Sco1 [191]. This property can play a role in distinguishing the molecular functions of the two human paralogs, for example, in the molecular recognition with different protein partners. Sco2 has been indeed found to be involved in other pathways distinct from Cu_A assembly, such as in the maintenance of cellular copper homeostasis [192] and in the p53-linked pathway [193]. The structural plasticity of Sco2, especially in its apo form, could be an essential requirement for taking part in the selection of the various protein pathways.

A single Cu(I)-binding site exists within the globular domain of Sco1 and Sco2, consisting of two cysteine residues within a Cx_3C motif and a conserved histidine residue (Fig. 4). Mutation of either Cys or His residues in Sco1 abrogates Cu(I) binding and results in decreased CcO activity [194]. Thus, the in vivo function of Sco1 correlates with Cu(I) binding [195]. These observations are consistent with the postulate that Sco1 mediates Cu(I) transfer from Cox17 to Cox2 (Fig. 4). Sco1 was indeed implicated in formation of the mixed valence Cu_A site of Cox2 as yeast Sco1 interacts with Cox2 [196] and as studies in organisms with a single Sco, such as *Bacillus subtilis*, suggest that the

copper in Sco1 is transferred to CcO [197]. According to this functional role, the Cu(I) ion in Sco1 is solvent-exposed and poised for ligand exchange transfer process. The structures of the metal-free Sco1 and of the Cu(I)Sco1 adduct are similar, with only loop 8 (which contains the copper-binding His) showing significant rearrangements [184]. The movement of this loop orients the Cu(I)-binding His residue in the proper orientation for copper binding. The structural dynamics of loop 8 suggest that it may be involved in the interaction with Cox2. Structural models of the protein complexes between Sco1 and Cox2 position indeed loop 8 of Sco1 in close contact with the metal-binding cysteine region of the two partners [198]. Accordingly, a series of conserved residues on the leading edge of this loop have been shown to be important for the Sco1/Cox2 interaction [185]. Copper(I) transfer likely occurs during a transient contact between Sco1 and Cox2, as it occurs for the Cox17 to Sco1 copper transfer. However, up to now direct evidence of the thiol ligand exchange mediating transfer of Cu(I) from Sco1 to Cox2 is lacking as a consequence of the high instability of the eukaryotic Cox2 domain in its apo state. Since Cu_A site in Cox2 contains two copper ions in a mixed valence Cu(I)/Cu(II) state, the question arises as to whether two copper(I) ions in the Cu_A site are sequentially derived from Sco1 via Cox17 or, since Sco1 can also bind a Cu(II) ion with high affinity in the same site of Cu(I) ion [195, 199], Sco1 proteins transfer both a Cu(I) and Cu(II) ion to form the mixed valent Cu_A site. This molecular mechanism has been recently investigated in a bacterial organism (discussed in detail later) showing that, even if the proteins involved in the bacterial Cu_A assembly are partially different with respect to the eukaryotic ones, a sequential insertion of two Cu(I) ions occurs yielding a reduced Cu_A site that can be converted to the oxidized, mixed valence site through electron transfer after the complex assembly [200]. In humans the molecular role of Sco2 in this mechanism is still quite elusive. However, it recently has been found that human Sco1 and Sco2 perform tightly coupled, but distinct step-specific functions in the maturation of the Cu_A site of CcO [189, 201]. A model has been proposed where human Sco2 acts upstream of human Sco1, and it is, at variance of human Sco1, indispensable for Cox2 synthesis [201]. Therefore, Cu(I)Sco2 interacts first with the newly synthesized Cox2 stabilizing it and triggering the recruitment of human Sco1 to the Sco2-Cox2 complex [201]. Then, after metallation of Sco1 by Cox17, copper is delivered to Cox2 sequentially by each Sco protein to form the Cu_A site, an event that results in the dissociation of the ternary complex and incorporation of the mature polypeptide into the nascent holoenzyme complex [201].

Mutations in Sco1 and Sco2 genes are responsible for mitochondrial diseases in humans associated to neonatal-onset hepatic failure and encephalopathy in the case of Sco1 [202] and to fatal infantile hypertrophic cardiomyopathy in the case of Sco2 [203]. All mutations produce respiratory deficiency associated with CcO assembly defects. All reported Sco2 patients carry E140 K missense mutation [203]. The solution structure of human Sco2 allowed us to rationalize at the molecular level the mutant misfunction [191]. Glu140, located in helix α1 and essentially not solvent exposed, is involved in a salt bridge with Lys143, which is disrupted in the E140 K mutant. Moreover, the introduction of a longer side chain in a buried region could locally destabilize side-chain packing between helix α1 and the facing β sheet. Since Glu140 is relatively close to the copper-binding Cys137, the structural rearrangements induced by the mutation could affect the copper-binding properties and protein stability, factors that both influence protein function. Sco1 mutations have only been reported in a single pedigree, with patients carrying a non-sense mutation on one allele and a P174L missense mutation in the second allele [202]. The solution structure of the P174L mutant in its Cu(I) form shows that Leu174 prevents the formation of a well-packed hydrophobic region around the metal-binding site and reduces its copper(I) affinity lower than Cox17 [181]. This translates into a defective copper metallation of Sco1 with the subsequent failure of Cu_A site maturation, thus establishing the basis for an inefficient assembly of the cytochrome *c* oxidase complex in Sco1 patients.

Formation of the Cu_B site is also dependent on accessory proteins for both the insertion of copper and heme groups. A copper-binding protein of the IM space, Cox11, is implicated in the formation of the Cu_B site of CcO. *S. cerevisiae* lacking Cox11 has impaired CcO activity and lower levels of Cox1 [204]. The critical fact suggesting a role for Cox11 in the Cu_B site formation was the finding that CcO isolated from *Rhodobacter sphaeroides cox11*Δ cells lacked Cu_B still having a Cox1 subunit containing both hemes, even if heme a_3 has an altered environment [205]. CcO in *R. sphaeroides* also has a Cu_A site, whose formation is unaffected in cells lacking Cox11. Thus, the absence of Cox11 appears to preclude Cu_B site formation without affecting delivery and insertion of heme *a* and Cu_A moieties.

Cox11 has a single transmembrane helix just downstream the N-terminal mitochondrial targeting sequence and a C-terminal domain protruding into the IMS which binds a single Cu(I) ion [206]. Three conserved Cys residues were candidate ligands for the Cu(I) ion. Mutation on any of these Cys residues indeed reduces Cu(I) binding as well as CcO activity, thus correlating Cu(I)-binding with in vivo function [206]. The structure of the globular domain of a Cox11 homolog from *Sinorhizobium meliloti* adopts an immunoglobulin-like β fold [207]. Two of the conserved

cysteines are located on one side of the β-barrel structure, while the third is located far from the others in the unstructured N-terminal region only five/four residues from the IM. Cox11 dimerizes upon Cu(I) binding to form a binuclear Cu(I) thiolate cluster at the dimer interface [206, 207]. A reasonable model for the copper site of the dimeric state of Cu(I)Cox11 contains one cysteine residue (Cys-100 or Cys-102) from each monomer acting as a monodentate ligand to one copper and one cysteine (Cys-100 or Cys-102) acting as a bridging bidentate ligand to both copper ions [207]. Indeed, the observed copper-copper distance of 2.7 Å found from EXAFS data is typical of double-bridged sulfur complexes, as found in other copper(I) chaperones with similar rearrangements. In this model the N-terminal cysteine would likely not be involved in copper binding but rather could be involved in an intermolecular disulfide bond with the corresponding cysteine of the other monomeric unit. Indeed, the same occurs in the structurally related Ig CD8-dimeric protein, which dimerizes through the S–S bond at the N-terminus. This intermolecular disulfide bond can be essential for the in vivo function, thus explaining the respiratory incompetence observed in the yeast Cys-111 (N-terminal cysteine) → Ala variant [206].

Cox17 is the specific copper donor to Cox11, as it results from in vitro and yeast cytoplasm expression system studies [177]. Then, the transfer of Cu(I) from Cox11 to the Cox1 Cu_B site buried 13 Å below the membrane surface may occur in nascent Cox1 chains extruded across the IM. Assuming that Cox11 directly transfers the metal to Cox1 subunit, the copper transfer could occur in a heterodimeric Cox1-Cox11 complex at the membrane interface. Therefore, it might be possible that the dimeric state of Cox11 may stabilize copper binding, protecting the copper ion until transfer to Cox1 occurs. Recently, it has been shown that Cox11 associates with the mitochondrial translation machinery, which can potentially signal to Cox11 the incoming translation of Cox1 [208, 209]. Concomitantly to the latter process, Cox11 may disrupt the dimer to form a heterodimer with Cox1 to insert co-translationally the copper ion in the Cu_B site. On the basis of a structural modeling study [198], we can also speculate that copper could be brought into the IM by mean of the Cox11 homodimer insertion. Indeed, aromatic conserved residues form a hydrophobic patch around the two copper ions that might promote interaction with the membrane driving the delivery of copper into Cox1. This is supported by the fact that predictions on membrane interaction surfaces indicate a putative binding site in the region of the dimer containing the above-mentioned aromatic residues [198]. However, a direct assay for detecting copper transfer from Cox11 to subunit I or any interaction between them remains to be shown. The use of in vitro bacterial systems exploiting co-expression and cell-free technologies has been recently developed to address several of the above biogenesis questions [210].

Interplay between cysteine redox chemistry and metal site biogenesis of copper enzymes in the IMS

Recent studies overthrow the previously held view that the presence or absence of disulfide bonds in proteins is determined by the cell compartment and that the simple ratio between reduced and oxidized glutathione always determines the status of intracellular thiol groups [211]. Instead, the specific nature of the proteins and their interactions with other proteins determine the redox states of Cys residues. Overall, the redox status of thiol groups is in equilibrium with the redox potential of the specific cellular compartment, but apparently this thermodynamic equilibrium involving disulfide formation and electron flow is also strongly modulated by protein-protein interactions so that, actually, only some pathways are allowed.

In this frame, it has been recently discovered that certain IMS proteins undergo oxidative folding processes to be trapped into IMS [212], thus achieving their native conformation (relying on the formation of intramolecular disulfides) once entered in the IMS. In particular, a protein machinery consisting of two proteins, Mia40 and Erv1, controls the protein import process and relies on a cascade of disulfide transfer reactions. This Mia40/Erv1-dependent protein import mechanism is strictly connected with the biogenesis of the copper sites of the CcO enzyme in IMS, as the mitochondrial copper chaperone Cox17 is a Mia40 substrate (Fig. 4). A mechanism of import of Cox17 into IMS was proposed [212]. Following its synthesis on cytosolic ribosomes, Cox17 traverses the outer membrane of mitochondria through the translocation pore formed by the TOM protein complex (Fig. 4). In IMS, it is initially bound by disulfide linkages to Mia40, a phylogenetically conserved receptor protein that contains a cysteine-rich domain [213]. Through reshuffling of the disulfide bonds, Cox17 is then released from Mia40 receptor in an oxidized, folded conformation (Fig. 4). The detailed molecular mechanism of the above import process has been recently characterized [214], and it was learned that: (1) Cox17 in the fully reduced state cannot efficiently be transformed into the partially oxidized state by oxygen alone for kinetic reasons; (2) Mia40 efficiently favors the formation of one of the two disulfides within the twin CX_9C motif of Cox17; (3) once the first disulfide (between Cys35 and Cys44) is formed, with the involvement of Mia40, the second disulfide bond between the two remaining cysteines of Cox17, which are now favorably positioned, can then be rapidly formed in vitro by oxygen. In vivo, the second disulfide may alternatively be formed by Erv1, which has been found to be physically linked to Mia40 under certain

conditions [215]. The recently solved solution structure of Mia40 as well as in vivo and in vitro mutational data are completely in agreement with this mechanism [214]. Cys 55 of the CPC active site of Mia40, critical for the intermolecular disulfide bond formation, is close to a characteristic hydrophobic cleft that functions as a substrate recognition/binding site, stabilizing initial non-covalent interactions that appropriately position the partially folded Cox17 substrate to form the first crucial mixed disulfide [214].

Cys-redox chemistry plays a crucial role not only in the import and folding of Cox17, which is the starting point of copper delivery towards CcO, but also in the following copper transfer steps towards Cu_A site of CcO. It has been shown indeed that Cu(I)Cox17 can simultaneously reduce the metal-binding Cys residues of Sco1 and transfer copper(I) to Sco1 and that the presence of copper(I) is thermodynamically necessary to drive the reaction [216] (Fig. 4). Sco proteins have a thioredoxin fold and were proposed to have not only the function of copper transfer, but also that of reducing the copper receiving Cys residues of the Cu_A site in CcO [184, 199, 217–219]. A crystal structure of human Sco1 where the metal ion is interacting with the disulfide bond formed by the two metal-binding cysteines strongly support the latter model [184]. This state might represent the transient complex, occurring just before the copper is transferred to the Cu_A site of the Cox2 subunit. Coupling of metal transfer to specific redox chemistry adds an additional level of control into the metal transfer pathway, warranting, in particular, a high metal specificity of this process for proteins capable of binding metals other than copper with a high affinity, as occurs for the Sco protein family [195, 220]. Such specificity is also carried out by means of Cu(I)Cox17 action toward metallation of oxidized apoSco1 with respect to oxidized apoSco2 as the electron transfer-coupled metallation does not occur with oxidized apoSco2 [216] (Fig. 4). Cox17 can therefore work in a selective way, thus being able to discriminate between two proteins with similar copper(I)-binding affinity. Since the redox potentials of the cysteines pairs in apoSco1 and apoSco2 are similar [181, 191], the different behavior of the two proteins toward redox chemistry has to rely on kinetic grounds, which might arise from the occurrence or absence of specific protein-protein interactions. Indeed, at variance with Cu(I)Cox17-Sco1 interaction, no Cu(I)Cox17-Sco2 adduct has been detected [216], thus suggesting that selective protein-protein interactions are crucial to position the disulfide bond of Sco1 in the correct orientation to facilitate the electron transfer reaction. This view is in agreement with previous findings showing that the metallation of Sco1, but not of Sco2, when expressed in the yeast cytoplasm, depends on the co-expression of Cox17, suggesting that Cox17 is a specific protein partner of Sco1 [195]. In conclusion, Sco1 and Sco2 proteins have different redox behavior when interacting with Cu(I)Cox17, but the same copper-binding capabilities and the same copper abilities to receive copper from Cox17 when they are in the apo reduced state (Fig. 4). Very recently, it has been suggested that Cu(I)Sco2, in a ternary complex with apoCox2 and Cu(I)Sco1, can also act as thiol-disulfide oxido-reductase to oxidise the copper-coordinating cysteines in Sco1 during Cu_A maturation [201].

The thiol-disulfide oxido-reductase function of Sco protein family has also been proposed to take place in bacterial organisms, which often contains Sco homologs in a variable number of duplicates [218]. The use of a thermophilic bacterial organism as a model for characterizing the molecular mechanism of Cu_A assembly recently allowed us to obtain the first direct evidence of Sco1 thioredoxin function towards oxidized apoCox2 [200]. Sco1 from *Thermus thermophilus* is indeed able to reduce the disulfide bond of the Cu_A center, thus allowing the Cu_A site to accept Cu(I) ions from a periplasmic Cu(I) chaperone (named PCu_AC). The latter, thanks to its copper-binding properties, is able to selectively and sequentially deliver two Cu(I) ions to apo-Cu_A giving rise to the native $Cu(I)_2$-CuA site in vitro [200, 221]. In this organism, it has also been found that Sco1 does not work as a copper chaperone in terms of transfer of both Cu(I) or Cu(II) ions [200]. The mechanism of bacterial Cu_A assembly therefore consists of a sequential insertion of two Cu(I) ions donated by a metallochaperone once the disulfide bond of the Cu_A center was kept reduced by a thioredoxin [200]. The affinities for copper of the three proteins are completely in agreement with the observed Cu_A-reconstitution process, having higher Cox2 site affinity than those of PCu_AC and Sco1, and PCu_AC higher than that of Sco1 [200]. In eukaryotes Sco proteins have a larger affinity for Cu(I) than bacterial Sco proteins [181, 216], and therefore they may act as both chaperones and thioredoxins.

In conclusion, living organisms could have developed an efficient and rapid mechanism of copper incorporation into the Cu_A site capable of handling the complex redox scenario of IMS. Cox17 is the origin of an electron cascade that exploits Sco1 and Sco2 proteins to obtain the reduction of the Cys residues in the Cu_A site. The fully oxidized state of apoCox17 containing three disulfides, which is produced in the first step of the electron cascade, can be reduced by GSH in the IMS and the protein be recycled (Fig. 4).

An oxidative Cys-dependent folding process is also strictly connected with the formation of the copper site of SOD1 enzyme in the IMS. It has been shown that only a very immature form of SOD1 lacking both zinc and copper can efficiently enter the mitochondria [123]. The conserved disulfide in SOD1 is essential for its enzymatic activity but

must be reduced to facilitate SOD1 entering in mitochondria [123]. Once inside the IMS, SOD1 is converted to an active holo enzyme through the same post-translational modifications seen with cytosolic SOD1. Indeed, like SOD1, also CCS has a dual localization in both the cytosol and the IMS of mitochondria [165]; it has been shown that high levels of CCS in the IMS promote the import of SOD1 protecting cells against oxidative damage [222]. How can CCS and SOD1 enter mitochondria without a mitochondrial targeting presequence? Very recent studies by Hell and co-workers [223] and by Kawamata and Manfredi [222] have shown that this process occurs through the disulfide relay system involving Mia40 and Erv1 proteins. The disulfide relay system of Mia40 and Erv1 drives the transport of CCS into IMS, presumably following a similar mechanism found in other Mia40 substrates as Cox17 and small Tim proteins. The latter proteins contain two conserved CX_3C motifs, which form two intramolecular disulfide bonds essential for small Tim function, i.e., the import and transfer of mitochondrial inner and outer membrane proteins [224]. It has been shown that Mia40 forms in the IMS disulfide intermediates with CCS, but not with SOD1 [223]. A transfer of disulfide bonds from Mia40 to CCS can thus occur, resulting in the trapping of CCS in the IMS. A mature copper-loaded CCS can afterwards be able to trigger the formation of disulfide bonds in SOD1, thus trapping the protein in the IMS. In the case of human SOD1, all four cysteines are critical for its retention in mitochondria [222]. The CCS cysteines used in disulfide relay are still unknown and may involve either the domain III CXC or domain I CXXC motifs described above. In conclusion, the modulation of the activity of the disulfide relay system has the ability to regulate the intracellular distribution of these proteins. Since the activity of the disulfide relay system itself is coupled to the respiratory chain through cytochrome *c*, which is a substrate of Erv1 [225], regulation of the respiratory chain might modulate, via the disulfide relay system, the levels of CCS and SOD1 in mitochondria to adapt them to the physiological requirement in mitochondria. Very recently, indeed, it has been observed that in mammalian cells, mitochondrial localization of CCS becomes detectable when Mia40 is co-expressed and that CCS mitochondrial content is affected by oxygen concentration, because CCS becomes detectable in mitochondria when oxygen concentration is reduced from 20% (high oxygen) to 6% (physiological oxygen) [222]. The physiological meaning of the enhanced CCS and SOD1 localization in mitochondria under lower oxygen remains to be determined. However, it can be suggested that when the cell is exposed to an increased oxidative environment, such as in the presence of high oxygen, CCS localizes primarily in the cytosol and retains SOD1 in this compartment [222]. A shift towards a more oxidative environment in the IMS, for example, during mitochondrial oxidative stress, will result in a more efficient disulfide relay import system, enhancing CCS and SOD1 import to scavenge mitochondrial superoxide. Therefore, CCS may function as a redox sensor that determines the localization of enzymatically active SOD1 in the cell compartment where it is most needed.

The CCS-driven mitochondrial uptake of SOD1 could also have important implications in SOD1-linked ALS. Indeed, it has been shown that the mitochondrial pool of ALS-SOD1 mutants causes damage to mitochondrial function and structure [226], and the mitochondrial uptake of ALS-SOD1 mutants is increased upon CCS overexpression [227]. This uptake of ALS-SOD1 mutants results in mitochondrial respiratory defects [228] and an acceleration in SOD1-linked motor neuron disease [227, 229].

Conclusions

Cellular biology of metal ions is becoming increasingly important because of its tremendous implications in medicine, as witnessed by a large number of proteins being involved in diseases linked to metal ion homeostasis. In this review we have analyzed some of the copper trafficking pathways involving metallochaperone and membrane proteins, like ATPases and permeases, which drive copper ion delivery towards final copper protein targets and modulate copper homeostasis in bacteria and eukaryotes. The molecular mechanism of action of this cellular machinery has been largely investigated in the last years showing that copper ions are specifically transported within the cell where its presence is requested. This machinery exploits high-affinity copper-binding proteins, which prevent the release of free toxic copper ions. Still, several issues of these pathways remain to be addressed. In particular, we still lack details of many aspects of the structure and molecular function of human copper-transporting ATPases. They are particularly relevant as they are involved in severe pathological dysfunctions. Future work on these ATPases will be also needed to yield deeper insights into the interplay between inherent molecular signals, like ATPase phosphorylation events, and the intermolecular interactions that govern their enzymatic activity and trafficking pathways in response to a range of physiological stimuli. Several aspects of mitochondrial copper homeostatic mechanisms are also not well understood. The mechanism of how copper reaches the matrix or is moved back in the IMS from the matrix needs further investigations. What is/are the functional role/s of several CX_9C containing proteins in CcO assembly and how mitochondrial copper metabolism affects cellular copper homeostasis are some of the remaining questions to be

answered. In this regard, cysteine redox chemistry has recently been shown to play a crucial role in influencing mitochondrial copper trafficking pathways. In particular, IMS is a compartment where Cys-redox chemistry and copper homeostatic processes seem to be strictly associated and likely related to disease development, as already suggested for fALS pathology. The molecular basis of all these processes are, however, still largely obscure, and their characterization needs further, extensive efforts in order to constitute a background for pharmacology and to have impact for more secure drug development.

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