Structure and chemistry of the copper chaperone proteins

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Major advances have been made in the past year towards an understanding of the structure and chemistry of copper chaperone proteins. Three-dimensional structures of Atx1, CopZ, yCCS, and hCCSdII were determined, and reveal a remarkable structural similarity between chaperones and target proteins. In addition, biochemical studies of CCS suggested that chaperones are required *in vivo* because intracellular copper concentrations are extremely low and also indicated that copper transfer occurs via a direct protein–protein interaction.

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Abbreviations

Atx1	copper chaperone for Ccc2
Ccc2	yeast P-type ATPase cation transporter
CCS	copper chaperone for SOD
CopZ	copper-operon protein
hAtx1	human Atx1
hCCS	human CCS
hCCSdII	hCCS domain II
hSOD1	human superoxide dismutase 1
Mnk4	fourth amino-terminal domain of Menkes disease protein
SOD1	copper, zinc superoxide dismutase
yCCS	yeast CCS
yCCSdI	yCCS domain I
yCCSdII	yCCS domain II
yCCSdIII	yCCS domain III
ySOD1	yeast superoxide dismutase 1

Introduction

Cells acquire essential metal ions such as copper for use in a variety of respiratory, metabolic and stress response enzymes [1]. These chemically reactive nutrients enter the cell and are ultimately incorporated into the appropriate active sites, but it is unclear how these processes are accomplished. One problem concerns the availability of intracellular copper. Another is the reactivity of the common oxidation states of copper, +1 and +2; both can readily react with dioxygen and related species. Some of these issues have come into clearer focus with the recent identification of metal-ion chaperones (or metallochaperones), which are part of the cellular machinery that ensures that the right metal ions get into the right active sites. These soluble, intracellular metal receptors protect, guide and deliver metal cofactors to specific target proteins. As discussed below, the chaperones exhibit chemistry quite distinct from that of typical copper enzymes.

The first protein demonstrated to be a copper receptor that interacts with a specific intracellular target was the yeast Atx1 protein. This copper chaperone binds Cu(I) and interacts directly with the amino-terminal domains of a P-type ATPase cation transporter, Ccc2, its physiological partner [2]. A related member of the copper chaperone family, the copper chaperone for superoxide dismutase (CCS), binds Cu(I) and delivers it to a specific target protein in the cytoplasm, namely copper, zinc superoxide dismutase (SOD1) [3,4•]. The bacterial copper-operon protein (CopZ) also binds Cu(I) and is thought to function in copper-transfer reactions [5]. The Cox17 protein functions in the assembly of cytochrome *c* oxidase and is essential for formation of the copper-loaded form [6–8]. Cox17 and CopZ might also have a metallochaperone function, but specific partners have yet to be identified. In this review, we examine advances made in 1999 towards understanding the structure and chemistry of metallochaperone proteins.

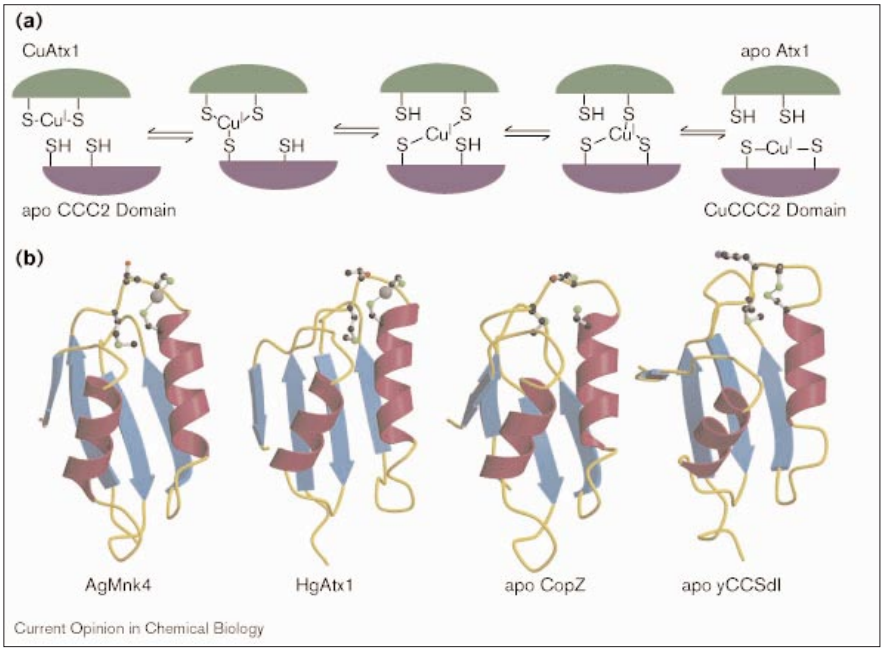
Atx1-like copper chaperones

The Atx1-like copper chaperones are highly conserved evolutionarily and the family is rapidly growing. After the yeast *ATX1* gene was shown to encode a metallochaperone in 1997, functional homologs were identified in a variety of organisms including bacteria [5], plants [9], mice [10], rats [11], dogs [12], and humans [13]. Mammalian Atx1 is present in many tissues, and is highly expressed in the choroid plexus of the mouse brain where it is proposed to participate in copper flux between the brain and cerebrospinal fluid [10]. In rats, the highest levels of Atx1 expression are associated with neuronal cell populations that sequester copper [14]. The Atx1 proteins are small polypeptides consisting of ~70 amino acids and have a distinguishing MXCXXC motif (using single-letter code for amino acids, where X is any amino acid) close to the amino terminus. This motif is also present in the amino-terminal repeats of the Menkes and Wilson disease proteins [15].

Antioxidant or metallochaperone?

The *ATX1* gene was first isolated by Lin and Culotta [16] in a screen that selected for suppressors of SOD1 deficiency. This antioxidant phenotype required overexpression of *ATX1* and was not observed when the Atx1 copy number was around the physiological level (~10^4 proteins per cell) [17]. Recent studies indicate that overexpression of the human Atx1 (hAtx1) homolog (encoded by the *HAH1* or *Atox1* gene) also protects cultured neuronal cells from stress induced by serum starvation or 100 μM hydrogen peroxide [18]. Examination of the antioxidant properties of yeast CuAtx1 *in vitro* reveals a stoichiometric, not a catalytic, reaction with oxidants such as superoxide [17]. This causes inactivation of the protein, probably through sulfhydryl oxidation and copper release. Sacrificial reactions of this type may explain the requirement for high copy number in the

Figure 1



Proposed copper-transfer mechanism from Atx1 to Ccc2 and comparison with other members of the structural family. **(a)** Proposed mechanism for facile copper-transfer within a docked complex of CuAtx1 (shown in green) and a physiological partner domain of Ccc2, a P-type ATPase cation transporter (shown in purple) [2]. **(b)** Members of the Atx1-like structural family. The structures are shown in equivalent orientations, and are color-coded according to secondary structure: α helices are shown in magenta, β strands are in blue, and loop regions are in yellow. For each protein, the residues corresponding to the methionine, threonine, and the two cysteines in the Atx1 MTCXXC motif are shown as ball-and-stick representations. Metal ions are shown as gray spheres. Protein Data Bank (PDB) accession codes are as follows: AgMnk4, 2AW0; HgAtx1, 1CC8; apo CopZ, 1CPZ; apo yCCSdI, 1QUP.

antioxidant phenotype and open the possibility that antioxidant activity might not be physiologically relevant in yeast. Further studies are required to resolve the proposed antioxidant roles of Atx1.

A clear physiological requirement for Atx1 was first established in the high-affinity iron uptake pathway of yeast [19], in which Atx1 was shown to function as a metallochaperone that delivers Cu(I) to Ccc2 [2]. Ccc2 is a P-type ATPase that transports copper into a trans-Golgi vesicle where it is taken up by the multicopper oxidase Fet3 [20]. The metallochaperone function was established in two ways: demonstration of specific Cu(I)-binding, and identification of an *in vivo* interaction with a genetically characterized partner protein in the high-affinity iron uptake pathway [2]. One mechanistic solution to the dilemma of how to transfer Cu(I) from one tight-binding site to another in a facile manner is shown in Figure 1a. In this mechanism, CuAtx1 is shown docking with one Atx1-like domain of Ccc2 so as to permit the close approach of metal-binding sites. As shown by spectroscopic studies, the site can adopt both two-coordinate and three-coordinate geometries at the metal center, leading to the proposal of a stepwise mechanism consisting of associative interchange reactions [2]. Recently, hAtx1 was shown to interact *in vivo* with some, but not all, of the amino-terminal domains of the human homologs of Ccc2, the Menkes and Wilson disease proteins, in a copper-dependent manner [21•,22•]. The ability of hAtx1 to interact with disease-causing mutants of the Wilson protein was diminished, suggesting that impaired copper delivery by hAtx1 can be a source of the disease for these particular mutations [22•]. Because hAtx1, Wilson protein and copper are all abundant in the brain, a role for hAtx1 in inherited neurodegenerative disease is anticipated in these studies.

Structure and mechanism

The high-resolution structures of HgAtx1 and apo Atx1 reveal a βαββαβ fold that is observed in a variety of proteins that bind inorganic ions or clusters: these include ferredoxin II, an iron–sulfur protein [23], bacterial mercury resistance protein (MerP) [24], and acylphosphatase [25•]. The solution structure of apo CopZ, a bacterial copper detoxification protein [26•] and of the apo and Ag forms of the fourth amino-terminal domain of Menkes disease protein (Mnk4) [27•] recently determined by NMR methods also exhibit this fold, as does the amino-terminal domain of the copper chaperone for yeast SOD1 (yCCSdI) [28•]. In HgAtx1 and Mnk4, the metal ion binds in a two-coordinate manner to cysteines located at a junction between the first loop and first helix. The conserved methionine does not come close enough to interact with the metal ion either in the domains of the transporters or in the copper chaperones. The structures of AgMnk4, apo CopZ and the amino-terminal domain of yCCS are compared to that of HgAtx1 in Figure 1b.

The structure of Atx1 (Figure 1b) corroborates the proposed mechanism (Figure 1a) in four ways [25•]. First, unlike in most copper proteins, the metal-binding site is not buried; instead, it is on the surface of the protein and thus is available for interaction with Cu(I) acceptor proteins. Second, the metal-binding loop provides only a few donor ligands, but these thiols stabilize metal ions that prefer low coordination numbers and typically undergo associative reaction mechanisms. Third, the loop is flexible

and can accommodate changes in coordination number. Finally, the structure reveals an array of positively charged surface residues that may be important for partner recognition. For example, mutation of Lys24 and Lys28 strongly diminishes the ability of Atx1 to interact with Ccc2 and to support iron uptake *in vivo* [17]. Lys65 is adjacent to the metal-binding residues and might modulate both metal–protein and protein–protein interactions.

The coordination environment of copper itself has yet to be observed in any of the crystal structures. X-ray absorption experiments on frozen solutions of Atx1 indicate that the copper is in the +1 state and is three coordinate with at least two thiolate sulfurs in the first coordination sphere. The identity of the third coordinating atom is not resolved. An exogenous thiol group and an amino-acid sidechain from the protein are both possible ligands [2]. The structure of HgAtx1 reveals a threonine sidechain oxygen atom located 3 Å from the metal ion. This residue could interact with the metal in the Cu(I) form of the protein. A variation on this coordination environment has been reported in spectroscopic studies of the Menkes protein. X-ray absorption studies of copper bound to a polypeptide comprising the six MXCXXC repeats of the Menkes protein revealed an average two-coordinate dithiolate Cu(I) environment [29]. These results are consistent with the original proposal that the Atx1 fold accommodates both two-coordinate and three-coordinate centers to facilitate copper-transfer reactions (Figure 1a), but provide no evidence of three-coordinate copper. Other Cu-thiolate environments may also be involved in copper-chaperone chemistry. Although not homologous to the above proteins, Cox17 binds Cu(I) through cysteine residues and forms an unusual sulfide-bridged copper dimer [8].

Prokaryotic relatives

CopZ is a copper-binding protein in the *cop* operon of *Enterococcus hirae* [30], which also encodes a copper-responsive repressor, CopY, and two copper transporting P-type ATPases, CopA and CopB [31]. CopZ was initially classified as a *trans*-acting metalloregulatory protein and activator controlling transcription of the *cop* operon [30], but other functions have also been proposed. NMR studies of the reduced form of apo CopZ [26•] reveal the same βαββαβ fold as Atx1, including a pair of cysteines in an exposed loop (Figure 1b). These studies further indicate that the loop is quite mobile in the absence of metal. The structure suggests that apo CopZ can accommodate the same type of Cu(I) binding and transfer reactions proposed for Atx1. Addition of copper salts to the sample led to some protein aggregation, however, and the metal site could not be resolved because of the loss of signals in the loop and in the adjacent helix. Solioz, Dameron and co-workers [5] have recently proposed another function for CopZ: delivery of copper to the CopY repressor. Addition of any form of Cu(I) *in vitro* abolishes the binding of CopY to the promoter and relieves the repression, allowing transcription initiation [32]. CopZ could also donate Cu(I) to the copper-efflux transporter, CopB, or receive it from the homologous uptake transporter CopA. Given the good homology between Ccc2, CopA, and CopB and the structural similarities between Atx1 and CopZ, a mechanism similar to that in Figure 1a is a possibility. The question of partnership remains open as no direct interaction between CopZ and any of the Cop proteins has yet been established.

Copper chaperones for SOD

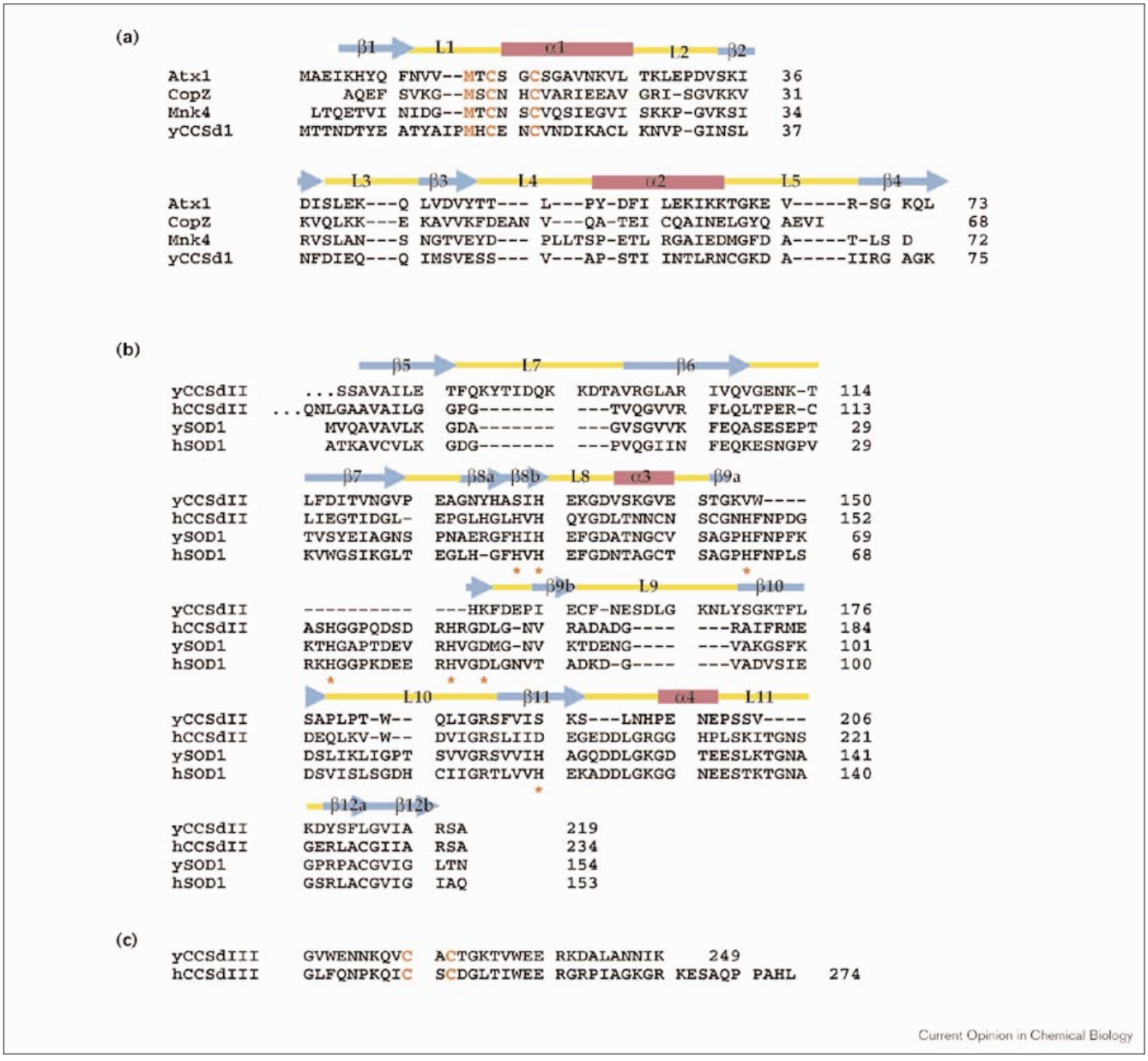
SOD1 is a cytosolic, homodimeric copper- and zinc-containing enzyme that catalyzes the disproportionation of superoxide to hydrogen peroxide and O_2, a key reaction in cellular antioxidant defense. As first shown by Culotta *et al.* [3], activation and formation of the copper-loaded state of SOD1 in yeast requires an accessory factor encoded by the *LYS7* gene. A homologous human gene was shown to complement the *LYS7* deficiency, and both the proteins encoded by the yeast and the human genes are known as copper chaperones for SOD1. In cells lacking CCS, SOD1 is expressed at normal levels, but the enzyme does not acquire the copper cofactor and is inactive [3,33].

CCS co-localizes with SOD1 in many mammalian tissues, including the brain and motor neurons in patients afflicted with amyotrophic lateral sclerosis [3,34,35]. Because mutations in SOD1 lead to one form of familial amyotrophic lateral sclerosis [36,37], proteins such as CCS that activate SOD1 might be relevant to understanding this disease. CCS can activate SOD1, but it cannot function in the place of Atx1 in the Ccc2-dependent high-affinity iron-uptake pathway described above [3]. Whereas the initial identification and characterization of CCS came from genetic approaches, biochemical and structural studies published in 1999 have begun to elucidate the molecular mechanisms of CCS.

Why are copper chaperones required?

Given that SOD1 binds copper with high affinity, the discovery of CCS raised several fundamental questions: why would a copper chaperone be required *in vivo*? Does CCS directly insert copper or does it play other roles in copper trafficking? Rae *et al.* have recently demonstrated that yCCS is necessary to activate SOD1 *in vitro* only when the concentration of free copper ions is severely limited [4•,38]. SOD1 is readily reconstituted *in vitro* without CCS if copper ions are present, but requires CCS if copper chelators are added to the solution. These results can best be explained if intracellular free copper concentrations are extremely limited. Using the known affinity of SOD1 for copper and the concentration of SOD1 *in vivo*, it can be shown that the free copper concentration in the cell is less than one attomolar, corresponding to less than one free copper ion per cell. This result suggests a physiological requirement for the metallochaperone: copper chaperones reserve copper from intracellular chelators so that it is available for incorporation into copper-containing enzymes. Another key finding of this study is that metal-ion delivery must occur by a direct-transfer mechanism. Because SOD1 is not activated in the presence of copper chelators, a mechanism in which copper

Figure 2



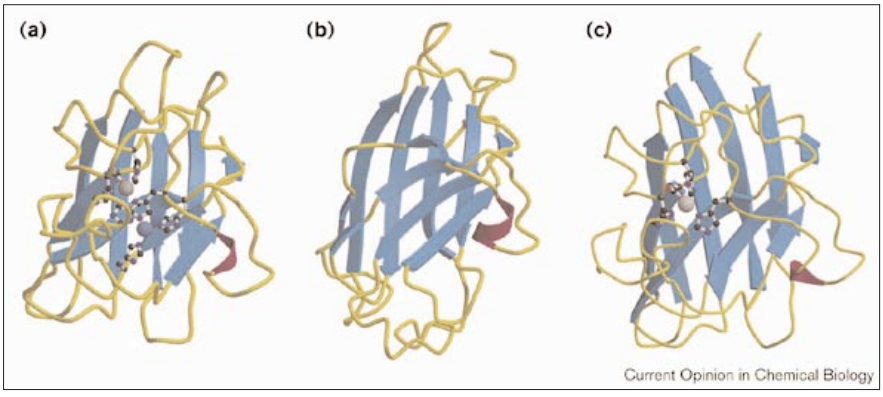
Sequence alignments of copper chaperones and their target proteins. **(a)** Structure-based sequence alignment of Atx1 with CopZ, Mnk4, and yCCSdI. The two cysteine residues involved in metal binding are shown in red. **(b)** Structure-based sequence alignment of yCCSdII with hCCSdII, ySOD1, and hSOD1. Metal ligands are denoted by red stars. For (a) and (b), the secondary structure elements for Atx1 and yCCSdII are shown above the alignments and color-coded as in Figure 1. **(c)** Sequence alignment of yCCSdIII and hCCSdIII. The two cysteine residues are shown in red.

is released by CCS into solution and then binds to SOD1 is unlikely. Interaction between CCS and SOD1 has been examined by *in vitro* column binding assays and *in vivo* immunoprecipitation experiments, which also suggest that the two proteins bind in a copper-independent manner [34].

Structure and biochemistry

Additional biochemical studies have addressed how CCS might transfer copper directly to SOD1. Examination of the yCCS and human CCS (hCCS) sequences (Figure 2) suggested that the chaperone comprises three domains: an amino-terminal domain that is homologous to Atx1 (domain I); a middle domain that is homologous to SOD1 (domain II); and a short carboxy-terminal domain with no detectable homology (domain III). According to proteolytic-protection studies on yCCS, these three regions do fold into distinct polypeptide domains [39•]. The functions of these yCCS domains were investigated *in vivo* by

Figure 3

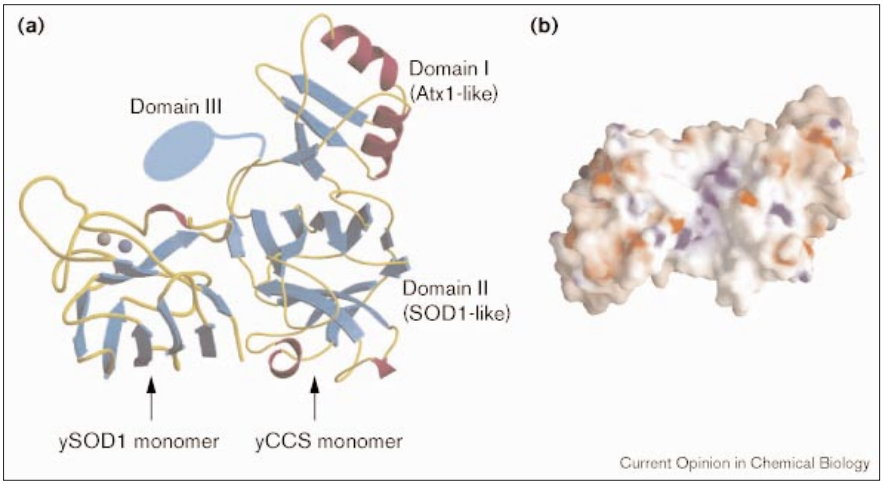


Structural comparison of SOD1 and the SOD1 domains of CCS. **(a)** Yeast SOD1 (PDB accession code 1SDY). **(b)** yCCSdII (PDB accession code 1QUP). **(c)** hCCSdII (PDB accession code 1DO5). The figures are color-coded as in Figure 1. The metal-binding sites are shown as ball-and-stick representations for ySOD1 and hCCSdII. The zinc ions are shown as gray spheres, and the copper ion is shown as a blue sphere.

deletion-mutagenesis experiments. Domain I was necessary for yCCS activity only under limiting copper concentrations, and was not interchangeable with Atx1. By contrast, domain III was absolutely required for SOD1 activation. Furthermore, mutation of two cysteine residues in domain III abolished yCCS activity, suggesting that these two residues, which are arranged in a CXC motif, might bind copper ions. Although domain II was necessary for activity, no evidence for metal binding by this domain was obtained.

The crystal structure of the apo form of yCCS determined by Lamb *et al.* [28•] revealed a dimer and established the folds of domain I and domain II [40]. The carboxy-terminal 27 residues, corresponding to domain III, were disordered in the structure. Domain I closely resembles Atx1 in overall structure (Figure 1b), although a number of basic surface residues that are proposed to be important in Atx1 recognition by its target, Ccc2 [17], are not present. The CXXC motif and metal-binding loop are conserved, suggesting that this domain plays a role in metal binding (Figure 1b, 2a). Domain II (Figure 3b) exhibits the same fold as its target enzyme SOD1 (Figure 3a), but lacks two loop-regions corresponding to the SOD1 zinc subloop and electrostatic channel loop, which form the SOD1 metal binding sites and active site channel. As a result, there is no metal-binding cavity in domain II (Figure 3b), consistent with the observation that only one of the metal ligands in SOD1 is conserved in yCCS (Figure 2b). The most striking feature of the yCCS structure is that the yCCS dimer interface, formed exclusively by the two domain II subunits, is remarkably similar to that in SOD1, both in size and in specific interacting residues. The dimer structure of apo yCCS in the crystal is interesting because this

Figure 4



Heterodimer and heterotetramer models for SOD1–CCS interaction. **(a)** Model of a possible heterodimer with secondary structure elements color-coded as in Figure 1. One monomer of ySOD1 is docked with one monomer of yCCS preserving dimer-interface interactions. There is no steric interference between the involved residues. The copper ion is shown as a blue sphere and the zinc ion is shown as a gray sphere in ySOD1. yCCSdIII is represented as a blue oval. **(b)** Alternatively, metal transfer could occur when a dimer of CCS interacts with a dimer of SOD1. Surface representation of hCCSdII, color-coded according to the electrostatic potential, is shown (blue positive, red negative). Because of the conformation of a loop that corresponds to the only structural element unique to CCS proteins as compared with SOD1 [41], four arginine residues are exposed at the CCS dimer interface (blue at center). This positively charged patch is a possible site for interaction between dimeric CCS and dimeric SOD1.

protein is a monomer in solution even at millimolar concentrations. Addition of Cu(I) apparently shifts the monomer/dimer equilibrium to favor the dimer state [39•]. These metal-induced changes in dimerization need to be taken into account as mechanisms are tested.

The crystal structure of domain II of hCCS has also been determined (Figure 3c) [41]. Unlike yCCS domain II, hCCS domain II does contain loops comparable to the SOD1 zinc subloop and electrostatic channel loop. These two loops form a zinc-binding site, similar to that in SOD1. The loop corresponding to the SOD1 electrostatic channel loop lacks many of the residues important for catalysis, however. Six of the seven ligands involved in SOD1 metal-binding are conserved in hCCS, the only exception being the replacement of a histidine, which coordinates the copper ion in SOD1, with aspartic acid (Figure 2b, 3a,c). This substitution apparently precludes copper binding to hCCS domain II. When this aspartic acid is mutated to histidine, a variant of hCCS that exhibits SOD1 activity is obtained [42]. The hCCS domain II structure also reveals a segment of loop 10 that is unique to the chaperones and is not found in SOD1 (Figure 2b). Finally, hCCS dimerizes in the same fashion as yCCS and SOD1. Some of the hCCS structural features were predicted by homology modeling [43].

Mechanistic implications

Taken together, the biochemical and structural data suggest that CCS is made up of three independent domains that have distinct functions. Domain I is likely to recruit copper, domain II facilitates recognition of SOD1, and domain III probably mediates copper delivery, perhaps in concert with domain I. In one possible mechanism, yCCS binds one Cu(I) per monomer, perhaps in a three-coordinate site at the interface between the metal-binding loop of domain I and the essential thiols of domain III [39•]. Although the domain III structure is unknown, it is clear from the yCCS structure that domains I and III could adopt a proximal orientation. Binding of Cu(I) induces a conformational change, detected as a decrease in the accessibility of domain III to trypsin cleavage in proteolytic time-course experiments. This metal-induced conformational change increases the propensity of yCCS to associate with itself and perhaps with apo SOD1 [39•]. The structures of yCCS and hCCS suggest possible mechanisms by which the chaperone recognizes and docks with SOD1. In one scenario, a monomer of CCS could interact with a monomer of SOD1, exploiting the highly conserved dimerization interfaces of both proteins to form a heterodimer (Figure 4a). In this model, the putative metal-binding site in domain I is ~40 Å away from the SOD1 copper-binding site; however, domain III might bridge this gap, enabling the CXC motif to directly release the Cu(I) into the SOD1 active site. Alternatively, a dimer of CCS could interact with a dimer of apo SOD1, employing the segment of loop 10 and the adjacent positive surface patch, neither of which are present in the SOD1 dimer.

The demonstration of multiple types of copper chaperone in a single cell and the high chelation capacity of the intracellular milieu raises the question of whether metallochaperones are required for other transition metals. There is no direct evidence for metallchaperones that deliver metals other than copper at this time. However, several small soluble proteins have been implicated in metal-ion delivery to or the activation of metalloenzymes. The UreE protein has been proposed to bind intraceulluar Ni(II) for transfer to urease apoprotein [44]. In the case of the iron-containing yeast ribonucleotide reductase R2, the Y4 protein is necessary for formation of the diiron-tyrosyl radical cofactor *in vitro* and *in vivo* [45•]. A significant number of proteins are required for iron–sulfur cluster assembly [46] and molybdenum-cofactor assembly including insertion of FeMoCo into nitrogenase [47]. Some of these appear to be heat-shock proteins, such as Ssq1p and Jac1p, and might function as molecular chaperones [48]. Molecular chaperones typically alter the folding state of the protein rather than insert metal ions. A series of other proteins, including NifS [49], are also required for assembly/maturation of the clusters by providing components such as sulfide. A role in folding has been suggested for the MelC1 protein, which is required for activation of the copper enzyme tyrosinase [50]. In the latter case, such proteins are better considered as molecular chaperones than metallochaperones. Furthermore, auxiliary proteins required for activation of metalloenzymes may in some cases involve other types of post-translational modification. Discriminating between a metallochaperone and other types of functions requires a demonstrated partnership between metal donor and acceptor proteins and evidence of metal protein interaction conducive to metal transfer for both partners.

Conclusions

Significant progress has been made toward understanding the biochemical and structural aspects of copper-chaperone function. One common theme that emerges from these studies is that Cu(I), rather than the more common oxidation state Cu(II), is employed in intracellular copper trafficking. All the copper chaperones characterized to date preferentially stabilize and exchange Cu(I). The properties and mechanisms of these proteins are still to be explored, but the discoveries that CCS mediates copper insertion into SOD1 via a direct interaction and that CCS is required because intracellular copper concentrations are very low help frame the metallochaperone function. The crystal structures of Atx1 and CCS have revealed that each chaperone is structurally similar to its partner protein. This extraordinary similarity may prove to be a general feature of metallochaperone/target-protein pairs.

Acknowledgements

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