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Copper Delivery by Metallochaperone Proteins

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ABSTRACT
Copper is an essential element in all living organisms, serving as a cofactor for many important proteins and enzymes. Metallochaperone proteins deliver copper ions to specific physiological partners by direct protein−protein interactions. The Atx1-like chaperones transfer copper to intracellular copper transporters, and the CCS chaperones shuttle copper to copper,zinc superoxide dismutase. Crystallographic studies of these two copper chaperone families have provided insights into metal binding and target recognition by metallochaperones and have led to detailed molecular models for the copper transfer mechanism.

Introduction

Copper plays a key role in all living organisms, serving as a cofactor for many proteins and enzymes involved in electron transfer, oxidase and oxygenase activities, and detoxification of oxygen radicals.[1] High concentrations of copper can be deleterious, however, leading to oxidative damage of proteins, lipids, and nucleic acids. Thus, intracellular copper concentrations must be rigorously controlled such that copper is provided to essential enzymes but does not accumulate to toxic levels. How this is accomplished for copper as well as for other essential yet potentially dangerous metal ions is not well understood and represents a central problem in bioinorganic chemistry. The importance of understanding how metal ions are handled within cells is underscored by recent research linking a number of human diseases to deficiencies in metal ion trafficking pathways. Diseases of copper metabolism include Menkes syndrome,[2] Wilson disease,[3] and familial amyotrophic lateral sclerosis (FALS).[4,5] Copper may also play a role in Alzheimer's disease[6] and prion diseases.[7] Abnormalities in iron and manganese metabolism have been implicated in other human diseases.[8]

The molecular details of intracellular copper trafficking have begun to emerge over the past few years with the discovery of copper handling proteins called copper chaperones or metallochaperones.[9−11] These soluble, cytosolic proteins deliver copper ions to specific target proteins via direct protein−protein interactions.[12] A metallochaperone protein is different from a molecular chaperone in that metallochaperones protect and deliver metal ions, whereas molecular chaperones facilitate protein folding.[13] Thus far, two metallochaperone-mediated copper delivery pathways have been characterized in detail (Figure 1). Both pathways, which are highly conserved between yeast and humans, start with copper import by the Ctr membrane transporters.[14−16] Once inside the cytosol, the chaperone Atx1[17] (Hah1 or Atox1 in humans)[18,19] shuttles copper to transport ATPases in the secretory pathway, and yCCS (*y*east *c*opper *c*haperone for superoxide dismutase, hCCS in humans)[9] delivers copper to copper,zinc superoxide dismutase (SOD1). In addition to yeast and humans, homologues of Atx1 and CCS have been identified in bacteria,[20] plants,[21,22] and other animals.[23−26]

Atx1 is a 73 amino acid protein that binds copper in the 1+ oxidation state[10] and delivers it to the P-type ATPase Ccc2 for translocation across intracellular membranes and loading into the multicopper oxidase Fet3 (Figure 1).[27] Fet3 is then localized to the cell surface, where it is proposed to oxidize Fe(II) to Fe(III) for transport across the plasma membrane.[28] Deletion of the gene encoding Atx1 in yeast cells results in defects in high-affinity iron uptake.[17] Expression of the human Atx1 homologue, Hah1, restores iron uptake, consistent with the presence of an analogous pathway in humans.[18] The human homologues of Ccc2 are the Menkes and Wilson ATPases, mutations in which are responsible for Menkes syndrome and Wilson disease,[29] and the human counterpart to Fet3 is ceruloplasmin, defects in which lead to abnormal iron metabolism.[30]

Atx1 and its homologues contain a conserved N-terminal MT/HCXXC (single-letter amino acid code where X is any amino acid) sequence motif that binds metal ions with the two cysteines. This motif is also present in the soluble, N-terminal regions of the target ATPases. Ccc2 contains two MT/HCXXC domains, whereas the Menkes and Wilson proteins each have six repeats of this domain. In the cell, Atx1 and the N-terminal repeat of Ccc2 have been shown to interact in a copper-dependent fashion.[10] In addition, direct and reversible copper transfer between Atx1 and this domain has been demonstrated in vitro.[31] Copper-dependent interactions between Hah1 and the Menkes and Wilson proteins have also been observed.[32,33]

In the second pathway shown in Figure 1, copper is acquired by the chaperone CCS and delivered to cytoplasmic SOD1, an antioxidant enzyme that catalyzes the disproportionation of superoxide to hydrogen peroxide and dioxygen by redox cycling of a copper ion.[34] Like Atx1, CCS binds Cu(I) rather than Cu(II).[35] In yeast, yCCS is encoded by the *LYS7* gene, deletion of which results in loss of SOD1 activity. SOD1 is still expressed at normal

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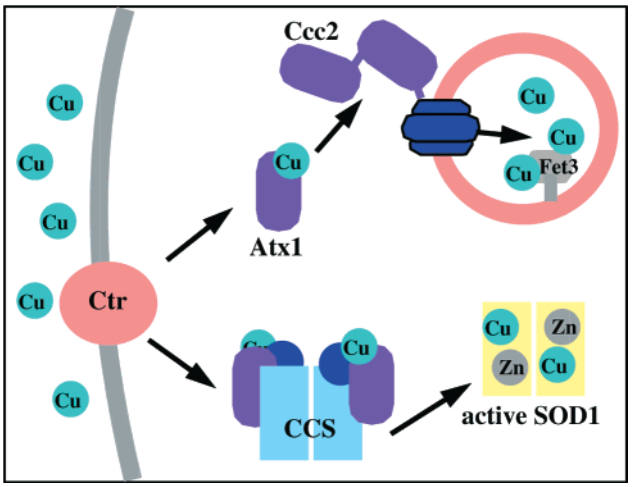

FIGURE 1. Two metallochaperone-mediated copper delivery pathways in yeast.

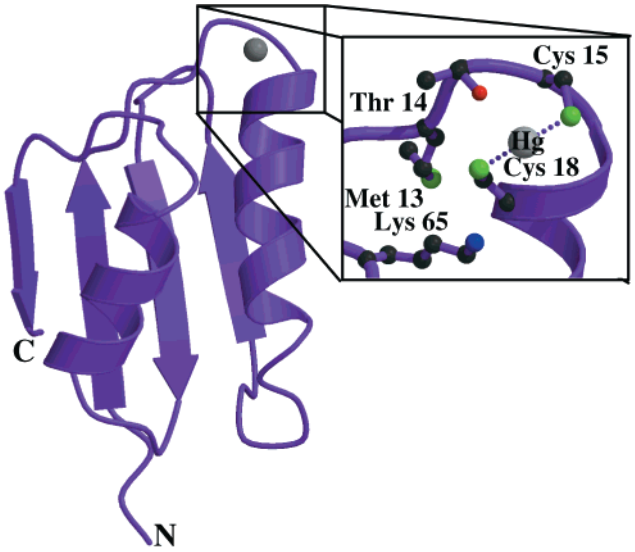

FIGURE 2. Structure of the Hg(II) form of Atx1. The enlargement shows the details of the Hg(II) binding site, including the conserved residues in the MT/HCXXC motif and an adjacent lysine residue.

levels in these yeast mutants but is inactive due to a defect in copper incorporation. SOD1 activity can be restored by expression of hCCS,[9] suggesting that the yeast and human forms of this metallochaperone employ similar mechanisms. Mutations in the gene for human SOD1 have been linked to FALS,[4,36] but how these mutations contribute to motor neuron degeneration and whether CCS plays any role in this process are not yet established. The CCS chaperones are significantly larger than the Atx1-like chaperones, with yCCS comprising 249 residues and hCCS 274 residues. The N-terminus of CCS contains the same MT/HCXXC sequence motif found in the Atx1-like chaperones and the N-termini of their target proteins. Like Atx1 and Ccc2, CCS and SOD1 interact directly.[35,37,38] CCS is necessary for SOD1 activation in vitro only if copper chelators are present, suggesting that copper chaperones are required in vivo because intracellular free copper concentrations are extremely limited.[35]

With the functions of these two copper chaperones clearly established by the genetic and biochemical data summarized above, the question of how copper delivery is accomplished on the molecular level has become the subject of intense research by a number of laboratories. Several years ago, my research group and our collaborators began to address the molecular mechanisms of copper delivery by Atx1 and CCS by using X-ray crystallography and biophysical techniques. In this Account, I review our structural work on these two classes of copper chaperones in the context of three broad questions. First, how do these chaperones bind copper specifically and in such a way as to facilitate a delivery function? Second, how does each copper chaperone recognize and interact with its physiological partner protein? Atx1 and CCS are not interchangeable but are very specific for their respective target proteins.[9] Third, what are the molecular details of the copper transfer process? The insights into these questions provided by our recent crystal structures of Atx1, Hah1, yCCS, and one domain of hCCS establish paradigms that should be applicable to other metallochaperone proteins.

Atx1-like Copper Chaperones

Metal Binding. Atx1, Hah1, and the other Atx1-like chaperones and target domains bind metal ions with the two cysteine residues in the conserved MT/HCXXC motif. This type of metal binding site was first structurally characterized with metal ions other than copper due to the instability of Cu(I) in aqueous solution. Structural evidence for coordination by the conserved cysteines initially came from the NMR structure of the fourth domain of the Menkes protein (Menkes4 domain) in the presence of Ag(I).[39] The Ag(I) ion is coordinated linearly by the two cysteine residues. A similar coordination environment is observed in the bacterial Hg(II) binding protein MerP, which also contains an MT/HCXXC motif.[40] Our 1.02 Å resolution X-ray structure of the Hg(II) form of Atx1 revealed that Atx1 binds metal ions in an analogous fashion (Figure 2).[41] The Hg(II) ion is coordinated by the two cysteine sulfur atoms with Hg···S bond distances of 2.33 and 2.34 Å and a S−Hg−S bond angle of 167°.

The overall structure of Atx1 comprises a $\beta\alpha\beta\beta\alpha\beta$ fold, similar to that observed for the Menkes4 domain and MerP. The two cysteines are located at the junction of the first β strand and the first α helix in the $\beta\alpha\beta\beta\alpha\beta$ fold, and the metal binding site is exposed at the protein surface (Figure 2). By contrast, the iron−sulfur clusters in the structurally similar ferredoxins and the catalytic copper sites in most oxidase and oxygenase enzymes are more buried. The chaperone metal binding site is thus uniquely suited to a delivery rather than a catalytic or electron-transfer function. We also determined the structure of the apo (metal-free) oxidized form of Atx1, in which the two cysteine residues form a disulfide bond.[41] The overall structure is nearly identical to that of the Hg(II) form, but oxidation of the two cysteines is accompanied by a significant conformational rearrangement of the metal binding loop. This form of Atx1 is probably not physi-

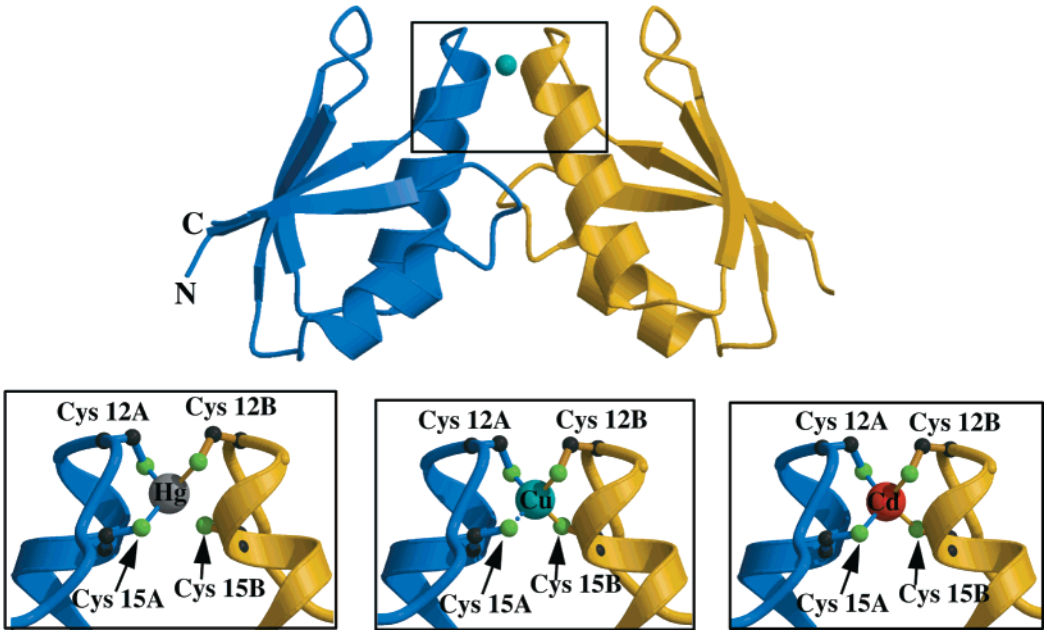

FIGURE 3. Structure of the human Atx1 homologue Hah1. The metal ion is coordinated by the conserved cysteine residues from 2 Hah1 molecules, shown in blue and yellow. The enlargements show the details of Hg(II), Cu(I), and Cd(II) binding.

ologically relevant, but the observed changes indicate that the metal binding loop is flexible.

Although the HgAtx1 structure established the participation of the two cysteines in metal binding, the details of Cu(I) coordination remained elusive. Crystals of the Cu(I) form of Atx1 did not contain an ordered, fully occupied copper site, a problem attributed to the crystallization conditions.[41] Since Atx1 can transfer Hg(II) as well as Cu(I) to a domain of Ccc2,[41] it seemed likely that the Cu(I) and Hg(II) coordination environments are similar. According to X-ray absorption spectroscopic data, however, the Cu(I) ion in Atx1 is coordinated by two sulfur ligands at 2.25 Å and a third ligand at 2.40 Å, suggested to be an oxygen or a nitrogen atom from another amino acid residue, the conserved methionine in the MT/HCXXC motif, or an exogenous thiol.[10] Since the sulfur atom of the conserved methionine is ~8 Å from the Hg(II) ion in the Atx1 structure (Figure 2), this residue is probably not a ligand.[41] By contrast, X-ray absorption spectroscopic data indicate a two-coordinate sulfur environment for copper bound to the six MT/HCXXC repeats of the Wilson protein[42] and either a two-coordinate[43] or a mixture of two- and three-coordinate sulfur geometries[44] for the six repeats of the Menkes protein. The solution structure of a bacterial Atx1 homologue, CopZ, was determined in the presence of copper, but the metal binding site could not be resolved.[45]

New insight into copper binding came from our crystal structure of the Cu(I) form of the Atx1 human homologue, Hah1.[46] In this 1.8 Å resolution structure, the Cu(I) ion is coordinated by cysteine residues from two separate Hah1 molecules, each of which closely resembles Atx1 in overall fold (Figure 3). The coordination is distorted tetrahedral with Cu···S distances of 2.3 Å for three of the cysteines and 2.4 Å for the fourth cysteine. Because these distances are not distinguishable within experimental error, the Cu(I) site can be described as either four-coordinate or three-coordinate with a weakly bound fourth ligand. Three-coordinate Cu(I)-thiolate centers in both model compounds and copper metallothionein exhibit Cu···S distances of 2.25−2.3 Å,[47,48] whereas biological four-coordinate Cu(I) thiolate centers have not been reported. The Hah1 structure strongly suggests that the third ligand detected in the Atx1 X-ray absorption spectroscopic data is a cysteine from an adjacent Atx1 molecule. The presence of an ordered copper binding site in these crystals as compared to the Atx1 crystals could be due to differences in crystallization conditions or improvements in anaerobic crystallization protocols. Alternatively, coordination by two Hah1 molecules might stabilize the Cu(I) ion against dissociation and subsequent disproportionation.

Two additional structures of Hah1 contribute to our understanding of how MT/HCXXC-containing domains define metal ion specificity. The structures of Hah1 in the presence of Hg(II) and Cd(II) were determined to 1.75 Å resolution.[46] In each structure, the metal ion is again coordinated by cysteine residues from two Hah1 molecules (Figure 3). The Hg(II) ion is three-coordinate, with three Hg···S bond distances of 2.3−2.5 Å and the fourth sulfur atom at 2.8 Å, a distance more consistent with a secondary bonding interaction.[49] The Cd(II) binding site is clearly four-coordinate. The four cysteine sulfur atoms are arranged in a tetrahedral fashion with Cd···S distances of 2.4−2.5 Å. This structure could explain why Hah1 incubated with Cd(II) does not interact with the Wilson protein.[32] The Cd(II) complex of two Hah1 molecules might be so stable that it prevents docking with and metal transfer to the Wilson protein. Since Cu(I) and Hg(II) do not readily form four primary bonds to thiolates, these forms of Hah1 can exchange ligands with the target domain, allowing metal ion transfer. A direct assay of

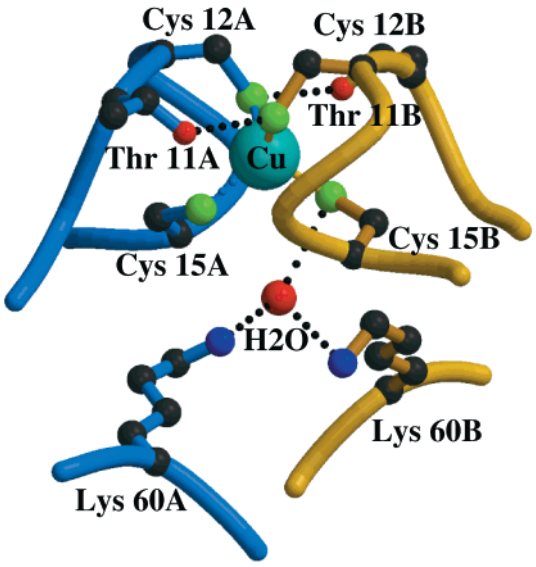

FIGURE 4. Intermonomer hydrogen bonding interactions at the Cu(I) binding site in Hah1. One monomer is shown in blue, and the other is shown in yellow.

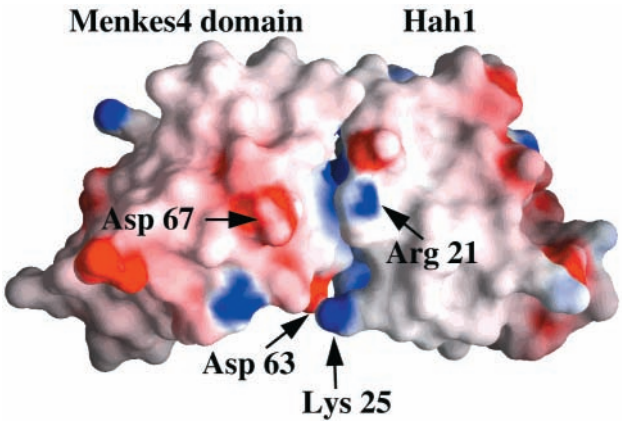

FIGURE 5. Surface representation of a model of Hah1 docked with the Menkes4 domain. The molecules are color coded according to electrostatic potential: red, −20 kT; white, 0 kT; blue, +20 kT. Asp 63 and Asp 67 from the Menkes4 domain are predicted to interact with positively charged residues from Hah1.

Cd(II) transfer, similar to that employed for Hg(II)[41] and Cu(I),[31] has not yet been reported.

Target Recognition. The Hah1 structures also provide a three-dimensional model for target recognition and docking. Since Atx1 and Hah1 are very similar to the Menkes4 domain in both overall fold and metal binding site, the observed Hah1 dimer can be considered as a model for the chaperone−target protein complex. The two Hah1 molecules contact one another primarily at the site of the shared metal ion.[46] The metal binding site is stabilized by a number of intermonomer hydrogen bonding interactions, the most significant of which involves the conserved threonine and cysteine residues in the MT/HCXXC motif. The side chain oxygen of the threonine interacts with the sulfur atom of the N-terminal cysteine on the opposite molecule (Figure 4). In addition, a lysine residue located near the metal binding site is hydrogen bonded to one of the C-terminal cysteine residues via an intervening water molecule.

Besides these interactions, the only other hydrogen bonds between the two monomers involve an arginine residue. This arginine and a number of lysines, most of which are conserved in Atx1, generate a positively charged patch on the surface of Hah1. On the basis of the Atx1 structure, we proposed that these positively charged residues participate in protein−protein docking by interacting with negatively charged residues on the surface of Ccc2.[41] In support of this proposal, mutation of these residues in Atx1 to alanine or glutamic acid impairs copper delivery to Ccc2.[50] The structural and sequential equivalents of these lysines in Hah1 are all located on the 2 α helices facing the second Hah1 molecule, further supporting a role in complex formation. Moreover, superposition of the Menkes4 domain coordinates on one of the Hah1 molecules in the dimer identifies two specific aspartic acid residues as possible candidates for interaction with the lysine residues (Figure 5).[46]

Metal Transfer Mechanism. The structures of Atx1 and Hah1 have allowed us to formulate a molecular picture of metal ion transfer between MT/HCXXC-containing domains. On the basis of spectroscopic studies of Atx1, it has been proposed that chaperone and target proteins dock with their MT/HCXXC metal binding loops in close proximity and exchange metal ions by the formation of two- and three-coordinate intermediates involving the cysteine residues from both proteins.[10] Several features of the Atx1 structure support this mechanism. The two-coordinate metal binding site exposed at the surface of the protein is well suited for docking with another protein and can accept a third ligand. In addition, the flexible metal binding loop can accommodate changes in coordination geometry. The Hah1 structures provide further mechanistic support by showing for the first time that two MT/HCXXC-containing domains can dock in such a way that direct metal transfer between the cysteines is possible. This finding, taken together with other features of the Hah1 structures, has allowed us to suggest a detailed mechanism for metal transfer. This mechanism, described below, advances our understanding of metal ion transfer between MT/HCXXC-containing domains to a new level by defining specific roles for conserved residues.

Metal ion exchange is predicted to proceed through the following intermediate structures (Figure 6). Prior to docking, the copper ion is probably bound to the chaperone in a two-coordinate geometry, similar to that observed in the Hg(II) form of Atx1. Upon docking, the N-terminal cysteine in the MT/HCXXC motif of the target domain donates a third ligand to the copper ion, forming a structure similar to the Hg(II) form of Hah1 (Figure 3). It is reasonable that this cysteine would bind first since it is housed on the flexible part of the metal binding loop, whereas the C-terminal cysteine is part of a more rigid α helix. The bond to the C-terminal cysteine on the chaperone then breaks, and a bond to the C-terminal cysteine on the target domain forms. This intermediate resembles the Cu(I) Hah1 metal binding site in which the bond to the C-terminal cysteine from the chaperone is slightly

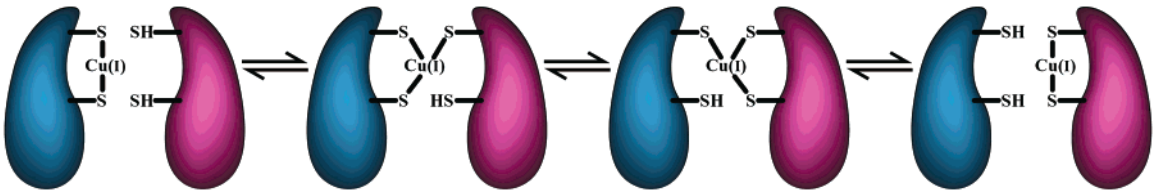

FIGURE 6. Proposed mechanism of copper transfer between Hah1 and a domain of the Menkes or Wilson protein (ref 46). Hah1 is shown in blue, and the target domain is shown in magenta.

longer (Figure 3). Ultimately, the copper ion ends up coordinated by the two cysteines in the target domain and the chaperone dissociates.

In addition to the direct participation of the cysteines, other conserved residues on or near the metal binding loop assist in this mechanism. Intermolecular hydrogen bonding interactions between the threonines and the N-terminal cysteines in the MT/HCXXC motifs from chaperone and target protein probably help position the two metal binding loops for transfer and may also partially neutralize the overall double negative charge on the three-coordinate intermediate. The threonine is conserved in almost all the chaperones and target proteins identified so far with the exception of the third repeat of the Menkes and Wilson proteins and the N-terminal domain of yCCS. In these domains, the threonine is replaced with a histidine, which could also form hydrogen bonds to the cysteines. In the Hah1 structure, hydrogen bonds link a lysine residue near the metal binding loop, a water molecule, and the conserved C-terminal cysteine from the opposite Hah1 molecule (Figure 4). Unlike the threonine, this lysine is only conserved in the chaperone proteins. Both repeats of Ccc2 and five of the six repeats of the Menkes and Wilson proteins contain a phenylalanine instead. The sixth repeat of the Menkes and Wilson proteins contains a proline. The Hah1 structures suggest a possible explanation for this difference. In the chaperone−target complex, this lysine might hydrogen bond directly to the C-terminal cysteine residue on the target protein, counterbalancing the negative charge from the two target cysteinates. This interaction would favor transfer of the metal ion since the chaperone would not have a similar interaction with the phenylalanine from the target protein.

Unresolved Issues. Although the crystal structures provide excellent models for metal transfer between Atx1 or Hah1 and the MT/HCXXC-containing domains of their target ATPases, further structural and biochemical characterization is required. In particular, crystallographic characterization of Atx1 or Hah1 in complex with one or more repeats of the target protein would reveal the specific residues involved in protein−protein docking. Another area in need of investigation is the role of the target protein MT/HCXXC repeats in the metal transfer mechanism. Do Atx1 and Hah1 interact with all or just some of the repeats in Ccc2 and the Menkes/Wilson proteins? It is not known whether all the repeats have the same function. For example, deletion mutagenesis studies on the Wilson protein indicate that the C-terminal repeats closest to the transmembrane channel are required for copper transport and cannot be replaced with the N-terminal repeats.[51] Similar studies of Menkes protein, however, imply that the N-terminal repeats are critical for function,[52] suggesting that the functional and structural organization of the six domains could differ between the two ATPases. Furthermore, the six repeats are predicted to have different electrostatic surfaces from one another, suggesting that not all are suitable for interaction with chaperone. The repeats might transfer copper between one another, utilizing a mechanism like that described above. Finally, copper-induced conformational changes in polypeptides comprising the six repeats have been reported. For both the Wilson[42] and Menkes proteins,[44] an alteration in secondary and tertiary structure is observed upon the binding of four copper ions. The interaction of this form of the protein with chaperone could be different from our structure-based model of chaperone complexed with one repeat. Clearly, structural characterization of multiple MT/HCXXC-containing repeats of the target ATPases both alone and in complex with the metallochaperone is an important goal for the future.

Copper Chaperones for SOD1

Metal Binding. Although the structure of CCS in the presence of copper has not yet been reported, the available structural, biochemical, and spectroscopic data suggest that the metal binding site includes multiple cysteine ligands. The CCS chaperones consist of three domains, an N-terminal Atx1-like domain (domain I), a middle domain that resembles the target enzyme SOD1 (domain II), and a small (∼30 residues) C-terminal domain (domain III) that has not been structurally characterized (Figure 7).[53,54] We have determined the X-ray structures of the first two domains of yCCS[53] and of hCCS domain II.[55] The 1.8 Å resolution structure of yCCS revealed that the N-terminal domain has the same fold as Atx1, including an exposed surface loop formed by the MT/HCXXC motif. In the crystal, the two cysteines are linked by a disulfide bond similar to that observed in apo oxidized Atx1.[41] The strong similarity of this loop to the metal binding loop in Atx1 and its homologues indicates that yCCS domain I probably binds metal ions.

Initial sequence comparisons suggested that CCS domain II would resemble SOD1.[9] This prediction turned out to be more correct for hCCS than for yCCS. Like SOD1, domain II from both yCCS and hCCS comprises an eight-stranded β barrel structure (Figure 8). In SOD1, the dinuclear copper,zinc center and the active site channel are formed by two extended loops, the zinc subloop and

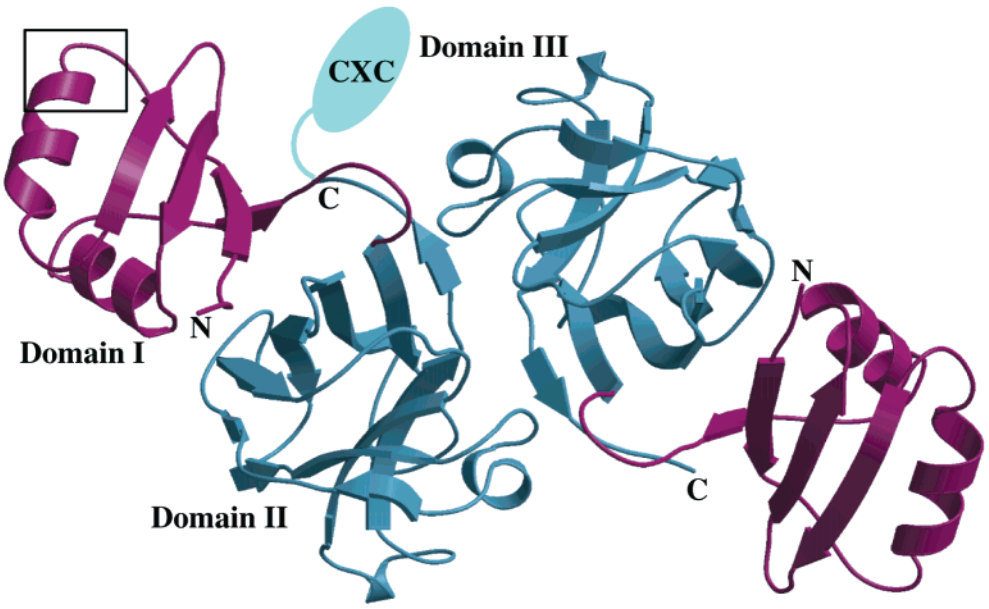

FIGURE 7. Structure of yCCS. The location of the MT/HCXXC sequence motif in domain I is denoted by a black box. Domain III was disordered in the crystal structure and is represented by the blue oval. The dimer interface is formed exclusively by residues from domain II.

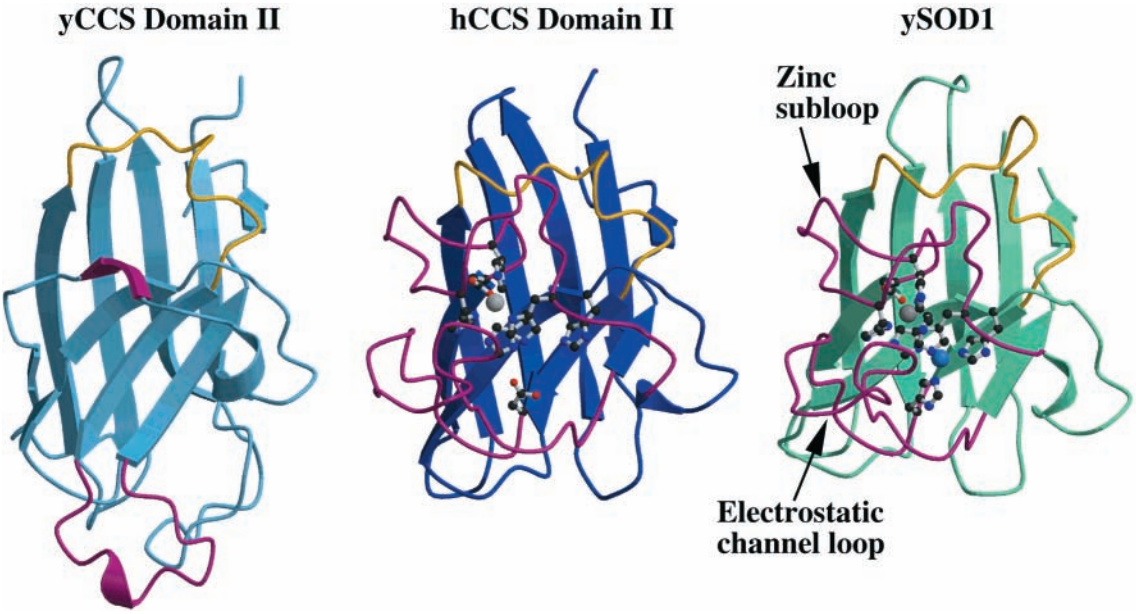

FIGURE 8. Comparison of yCCS domain II, hCCS domain II, and a monomer of yeast SOD1. The zinc subloop and electrostatic channel loop in SOD1 and the corresponding loops in yCCS and hCCS are shown in magenta. The loop shown in yellow is the only structural element unique to the two chaperones. Zinc ions are shown as gray spheres, and the copper ion in SOD1 is shown as a blue sphere. Coordinating residues are shown as ball-and-stick representations.

the electrostatic channel loop.[56,57] Domain II of yCCS lacks these two loops as well as six of the seven metal ligands found in SOD1.[53] Consequently, this domain in yCCS probably does not bind metal ions. By contrast, the two loops are structurally conserved in hCCS domain II and form two potential metal binding sites. The site equivalent to the SOD1 zinc site is occupied by a zinc ion in the crystal, but copper is not observed in the second site (Figure 8).[55] The substitution of aspartic acid for one of the histidines that binds copper in SOD1 might account for this difference. Interestingly, mutation of this aspartic acid back to histidine converts hCCS to an active SOD1 in vivo, indicating that copper can bind at this site.[58] Nevertheless, since yCCS domain II does not contain a metal binding site and hCCS domain II does not readily bind copper, it is unlikely that this domain binds copper for transfer.

In addition to domain I, domain III could be involved in metal binding. Not only are these 30 residues required for CCS function, but the ability to bind metal ions is suggested by the presence of a CXC sequence motif, both cysteines in which are necessary to activate SOD1.[54] One possibility is that domains I and III together generate the copper binding site. Spectroscopic data for hCCS and

tomato CCS (tCCS) in the presence of Co(II) are consistent with ligation by three or four cysteines.[22] Since tCCS only contains the four cysteines from the MT/HCXXC and CXC motifs, these data suggest that the metal binding site is located between the two domains. According to the Co(II) binding study and recent X-ray absorption spectroscopic data,[59] a second metal binding site is present in hCCS. This observation is consistent with the fact that yCCS can be reconstituted with both one and two copper ions.[54] The X-ray absorption data indicate the presence of a Cu···Cu vector at 2.7 Å and three sulfur ligands at 2.26 Å. From this analysis, a cysteine-bridged dinuclear copper cluster coordinated by the four cysteines from domains I and III was proposed.[59] A site involving simultaneous ligation by the cysteines from both domains is compatible with the yCCS crystal structure which predicts that domain III is located proximal to domain I (Figure 7).

Target Recognition. Target recognition by Atx1 and Hah1 is proposed to involve conserved charged residues on the protein surfaces and interactions between the chaperone and target domain MT/HCXXC metal binding loops. Although yCCS domain I resembles Atx1 and Hah1, recognition of SOD1 is likely to occur in a different fashion for several reasons. First, SOD1 does not contain an MT/HCXXC motif, so metal coordination and hydrogen bonding between two MT/HCXXC metal binding loops cannot occur. Second, the positively charged surface residues found in Atx1 and Hah1 are not conserved in yCCS domain I, with the exception of the lysine residue located adjacent to the metal binding loop. Finally, Atx1 and Hah1 recognize domains that are structurally similar to themselves. If structural similarity between metallochaperone and target protein is important, then recognition of SOD1 by CCS should employ domain II which resembles SOD1. This hypothesis is consistent with the observation that in vivo, yCCS domain I cannot substitute for Atx1, but Atx1 can substitute for yCCS domain I.[54] It is probable that yCCS domain I cannot replace Atx1 because it lacks specific surface residues that are important for interaction with Ccc2. The Atx1/yCCS domain II chimeric protein would recognize SOD1, however, if domain II rather than domain I mediates the protein−protein interactions.

The yCCS and hCCS structures suggest how domain II might facilitate target recognition. In both structures, CCS is a homodimer with the dimer interface formed exclusively by residues from domain II (Figure 7). Remarkably, the dimer interface is very similar to that in SOD1. Not only do the same secondary structure elements interact in the CCS and SOD1 dimers, but the dimer interfaces are approximately the same size, and specific interactions are conserved.[53,55] For example, the SOD1 dimer interface includes four strong main chain hydrogen bonds. The sequentially identical residues form the same hydrogen bonds in both yCCS and hCCS.

The conserved dimer interface could promote target recognition in two ways. First, CCS and SOD1 could interact as dimers, forming a dimer of dimers for metal ion insertion. One structural feature that is present in both yCCS and hCCS but not in SOD1 is a positively charged surface patch near the dimer interface.[12,55,60] This patch comprises several arginine residues that are exposed due to the substitution of a single tryptophan in CCS for a four-residue sequence in SOD1. As the only structural element unique to the chaperones, the loop containing this tryptophan and the resulting positively charged surface could be important for docking with SOD1. Alternatively, this region could participate in interactions with domain III. A model for how this surface patch might interact with SOD1 to form a dimer of dimers has been proposed.[60] Such a model is consistent with the observation that both CCS and SOD1 are dimeric in solution. SOD1[61] and hCCS[38] always exist as dimers, and yCCS is dimeric in the presence of copper.[54]

The second way in which the conserved dimer interfaces could facilitate target recognition is by allowing the formation of heterodimers consisting of one monomer of CCS and one monomer of SOD1. Comparison of the structures shows that a heterodimer could form without steric interference between the residues at the interface.[53] In addition, there is precedent for heterodimer formation between different SOD1s.[62,63] This model resonates well with the identification of an iron assembly protein for the yeast ribonucleotide reductase Y2 protein.[64] Both Y2 and its putative chaperone protein, Y4, are dimeric[64] and have been shown to form heterodimers.[65] Recent data indicate that CCS can, indeed, form a heterodimer with SOD1.[66,67] Complex formation between apo and copper-loaded yCCS and yeast SOD1 was investigated for both wild-type SOD1 and a mutant SOD1 in which copper ligand His 48 has been replaced with phenylalanine.[66] According to gel filtration chromatography, analytical ultracentrifugation, and chemical cross-linking experiments, yCCS and the mutant SOD1 form a complex with the correct molecular weight for a heterodimer. The heterodimer is also formed with wild-type SOD1 but is less stable. Notably, heterodimer formation is facilitated by the presence of zinc but is independent of whether copper is bound to yCCS. Higher order oligomers, such as a dimer of dimers, were not detected by any of these techniques.[66]

Metal Transfer Mechanism. Copper insertion is likely to occur via the observed heterodimeric complex for several reasons. First, SOD1 activation by copper-loaded yCCS requires zinc, and zinc promotes heterodimer formation.[66] Second, mutations at the dimer interfaces of either CCS or SOD1 prevent SOD1 activation in yeast cells.[68] Finally, the heterodimer formed with wild-type SOD1 is less stable than that formed with the mutant, consistent with a transient docked complex that dissociates after copper transfer. If a model of the heterodimer is generated (Figure 9), the MT/HCXXC loop in domain I is positioned ~40 Å from the SOD1 copper site. Contacts between domains I and II are minimal, and the seven-residue linker region is potentially flexible, suggesting that domain I could move toward the docked SOD1 monomer. A conformational change may not bring domain I close enough to directly insert metal into the SOD1 active site, however. This problem could be solved if domain III were to bridge the gap and use its CXC motif to shuttle the

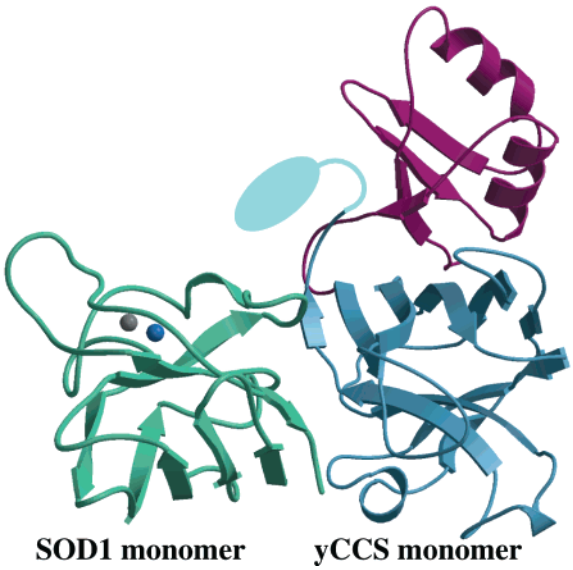

FIGURE 9. Model of a heterodimer formed by one monomer of yCCS and one monomer of yeast SOD1. The two monomers are docked, preserving key dimer interface interactions observed in the yCCS and SOD1 homodimers. In SOD1, the zinc ion is shown as a gray sphere, and the copper ion is shown as a blue sphere.

copper from the putative metal binding site in domain I to the SOD1 active site. This type of mechanism is supported by the spectroscopic data, suggesting that cysteines from both domains I and III are involved in metal binding[22,59] and by the absolute requirement of domain III for CCS activity.[54]

Unresolved Issues. Although the crystallographic, biophysical, and genetic data have greatly advanced our understanding of copper delivery to SOD1 by CCS, many questions remain unanswered. The details of copper binding, including stoichiometry and coordination geometry, are not clear. A crystal structure of the copper-loaded form of CCS would address these issues and would likely establish the fold of domain III. Structural characterization of the heterodimer would reveal specific residues involved in chaperone−target protein complex formation and elucidate molecular details of the copper transfer process. Another important question is whether CCS/SOD1 interactions differ for FALS-related SOD1 mutants. A number of FALS mutations in SOD1 are located near the dimer interface.[69] Investigations in this area could lead to the development of new therapeutics.

Conclusions

The structural, biophysical, and biochemical studies of Atx1, Hah1, yCCS, and hCCS summarized here have provided the first steps toward understanding intracellular copper delivery on the molecular level. Many issues remain unresolved, however. In addition to the specific questions detailed above for each class of chaperone, it is not clear how the copper chaperones initially obtain copper. Possible mechanisms include interactions with the Ctr membrane transporters, with proteins such as metallothionein or yet to be identified factors, and with small intracellular chelators such as glutathione. Another outstanding issue is the possible existence of other metallochaperones. For copper, several proteins have been implicated in the assembly of cytochrome *c* oxidase.[70−73] Proteins involved in nickel,[74] iron,[64] iron−sulfur,[75,76] molybdenum,[77] and iron−molybdenum[78] cofactor assembly have also been identified. Direct delivery of metal ions by these proteins has not yet been demonstrated, and some may act as molecular rather than metallochaperones. Nevertheless, future studies of these proteins are likely to be enhanced by insights gained from the Atx1 and CCS copper chaperone work. We predict that target recognition of and metal transfer to oligomeric metalloenzymes might occur by chaperone-mediated mechanisms similar to that proposed for CCS. Testing of this prediction and extending our knowledge of metallochaperone structure and function constitute key directions for future research.

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