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Cu(I) Affinities of the Domain 1 and 3 Sites in the Human Metallochaperone for Cu,Zn-Superoxide Dismutase

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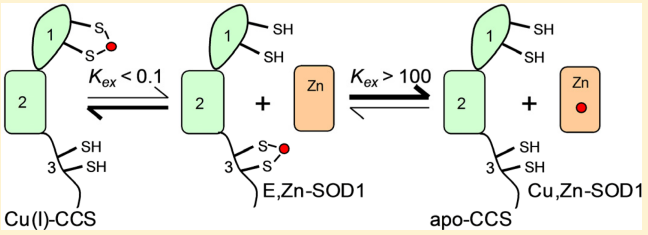
Supporting Information

ABSTRACT: The delivery of copper by the human metallochaperone CCS is a key step in the activation of Cu,Zn-superoxide dismutase (SOD1). CCS is a three-domain protein with Cu(I)-binding CXXC and CXC motifs in domains 1 and 3, respectively. A detailed analysis of the binding of copper to CCS, including variants in which the Cys residues from domains 1 and 3 have been mutated to Ser, and also using separate domain 1 and 3 constructs, demonstrates that CCS is able to bind 1 equiv of Cu(I) in both of these domains. The Cu(I) affinity of domain 1 is approximately 5×10^{17} M^{-1} at pH 7.5, while that of domain 3 is at least 1 order of magnitude weaker. The CXXC site will therefore be preferentially loaded with Cu(I), suggesting that domain 1 plays a role in the acquisition of the metal. The delivery of copper to the target occurs via domain 3 whose structural flexibility and ability to be transiently metalated during copper delivery appear to be more important than the Cu(I) affinity of its CXC motif. The Cu(I) affinity of domain 1 of CCS is comparable to that of HAH1, another cytosolic copper metallochaperone. CCS and HAH1 readily exchange Cu(I), providing a mechanism whereby cross-talk can occur between copper trafficking pathways.



A copper metallochaperone is responsible for activating Cu,Zn-superoxide dismutase in eukaryotes (designated SOD1 in humans and Sod1 in *Saccharomyces cerevisiae*), the only enzyme that is known to acquire copper in the cytosol.[1−11] This involves copper delivery and the formation of an essential disulfide bond in the target.[9−11] Both Cu,Zn-superoxide dismutase and its metallochaperone are also localized to mitochondria,[12] and to the nucleus where they are required for copper sensing by Mac1.[13] Understanding the activation of SOD1 has particular importance given that copper mishandling has been linked to a range of disorders, including Alzheimer's disease (AD), with mutations in SOD1, which can lead to misfolding, resulting in amyotrophic lateral sclerosis.[14−17] The copper metallochaperone for Cu,Zn-superoxide dismutase, CCS in humans and Ccs1 in *S. cerevisiae*, has three domains (Figure 1A).[3,6,7,18,19] Domain 1 (D1) has a ferredoxin-like fold as found for the other human cytosolic copper metallochaperone, HAH1 (Atx1 in *S. cerevisiae*), that is involved in the delivery of Cu(I) to the secretory pathway.[6,18−21] HAH1 and D1 of CCS both bind Cu(I) via a CXXC motif. Domain 2 (D2) of CCS is structurally similar to the target enzyme[18,22] and is the main region of interaction between these partners.[19] CCS contains a Zn(II) site in D2 at the same location as that of SOD1,[22] which is absent in Ccs1,[18] but does not bind copper.[22] Domain 3 (D3) is relatively short and contains a CXC Cu(I)-binding sequence.[3] Apo-CCS is a dimer, whereas apo-Ccs1 is monomeric, with alterations in quaternary structure observed for both proteins upon binding Cu(I).[3,23,24] The interaction with the target enzyme is thought to involve heterodimeric complexes (Figure 1A),[19,23,25,26] although a CCS−SOD1 heterotetramer has been suggested.[24]

The functional importance of the domains of CCS and Ccs1 has been studied.[3,24,27] The transfer of copper from CCS to SOD1 is possible in vitro in the absence of the D1 Cys residues,[24] but the CXXC motif is essential for the in vivo activation of SOD1.[27] Domain 1 of Ccs1 is required in *S. cerevisiae* only when copper is limiting,[3] and the absence of the CXXC motif of Ccs1 does not preclude Sod1 activation, although the influence of copper availability has not been tested.[28] Domain 3 is essential for Cu(I) transfer and disulfide bond formation in the target enzyme in humans and *S. cerevisiae*.[3,27] Both of the Cu(I)-binding domains of CCS must play a role in the delivery of Cu(I) to SOD1, but the mechanism of this process is not understood. It has recently been reported that SOD1 has a 10-fold higher affinity for Cu(I) than CCS loaded with 1 equiv of Cu(I), providing a thermodynamic driving force for copper transfer.[29] In this work, we show that both D1 and D3 of CCS can bind 1 equiv of Cu(I) and that D1 has a significantly higher affinity than D3 for the metal, consistent with a role in copper acquisition. The Cu(I) affinity of D1 of CCS is similar to that of HAH1, and the proteins readily exchange copper. Insight into copper trafficking in humans and particularly the roles of the copper-binding sites in domains 1 and 3 of the metallochaperone for SOD1 is provided.

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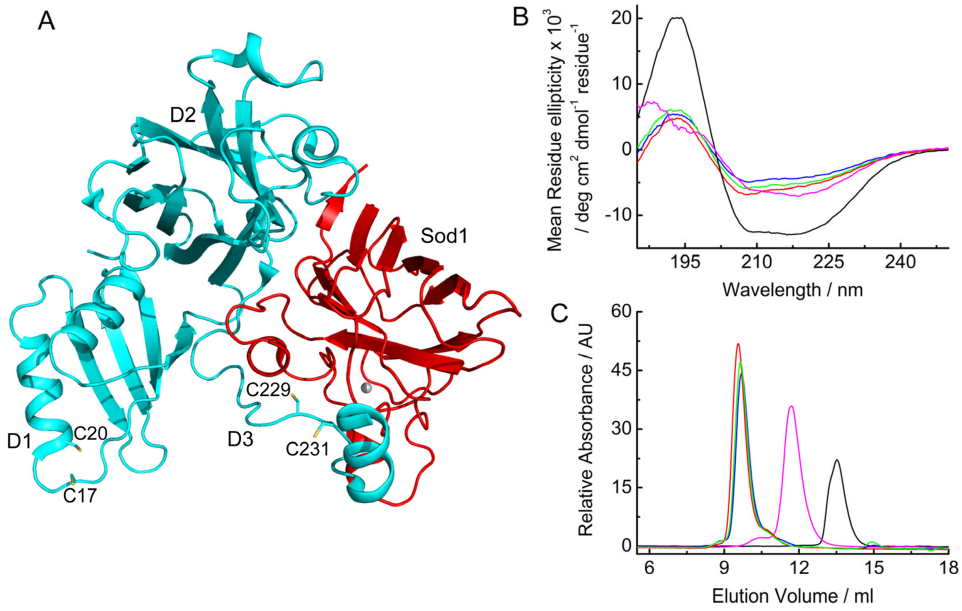

Figure 1. Structure and oligomeric state of wild-type CCS, the Cys to Ser variants, D1-CCS, and Ccs1. (A) Crystal structure (Protein Data Bank entry 1jk9) of the complex of apo-Ccs1 (cyan) with copper-free Sod1 (E,Zn-Sod1, red) from *S. cerevisiae*.[19] The side chains of the Cys residues of the CXXC and CXC motifs of D1 and D3, respectively, are shown as sticks, and the zinc ion is shown as a gray sphere. (B) CD spectra of apo-WT-CCS (blue line), apo-C22S/C25S-CCS (green line), apo-C244S/C246S-CCS (red line), apo-D1-CCS (black line), and apo-Ccs1 (magenta line) in 100 mM potassium phosphate (pH 6.5) (all at 0.5 mg/mL). (C) Analytical gel filtration chromatograms of apo-WT-CCS, apo-C22S/C25S-CCS, apo-C244S/C246S-CCS, apo-D1-CCS, and apo-Ccs1 (the colors of the lines correspond to the colors in panel B) for samples (100 μM when injected) in 20 mM Mes (pH 6.5) and 200 mM NaCl. The absorbance was measured at 280 nm except for that of D1-CCS (240 nm), and the values for Ccs1 have been divided by 3.

MATERIALS AND METHODS

Cloning and Mutagenesis. The gene for wild-type CCS (WT-CCS)[30] was cloned into pET29a via the NdeI and BamHI sites, giving pET29a_WT-CCS. Cys to Ser mutations in the D1 $C^{22}XXC^{25}$ (C22S/C25S-CCS) and D3 $C^{244}XC^{246}$ (C244S/C246S-CCS) motifs were prepared using Quikchange mutagenesis (Stratagene) and the primers listed in Table S1 of the Supporting Information, giving pET29a_C22S/C25S-CCS and pET29a_C244S/C246S-CCS, respectively. The DNA encoding residues 1−79 of CCS, corresponding to D1 of CCS (D1-CCS), was cloned (primers listed in Table S1 of the Supporting Information), into the NdeI and BamHI sites of pET29a to generate pET29a_D1-CCS. The gene for WT *S. cerevisiae* Ccs1[30] was cloned into pET29a using the NdeI and BamHI sites of pET29a (pET29a_WT-Ccs1) and the primers listed in Table S1 of the Supporting Information.

Expression and Purification of WT-CCS and the Cys to Ser Variants. A modified version of a previously reported protocol[31] was used for the purification of CCS. *Escherichia coli* BL21(DE3) transformed with either pET29a_WT-CCS, pET29a_C22S/C25S-CCS, or pET29a_C244S/C246S-CCS was grown in LB medium containing 100 μM Zn(II) at 37 °C until an OD_{600} of 0.6−0.8 was reached. Protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside, and cells were incubated for a further 6 h before being harvested, with pellets stored at −20 °C. Cells were resuspended in 20 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5) containing 4 mM ethylenediaminetetraacetic acid (EDTA) and 4 mM dithiothreitol (DTT), sonicated, and centrifuged at 35000*g* for 20 min. The supernatant was diluted 4-fold with Milli-Q water (Millipore) prior to being loaded onto a DEAE Sepharose FF column (~40 mL, GE Healthcare) and eluted with a linear NaCl gradient (0 to 300 mM) in 5 mM Tris (pH 7.5) containing 1 mM EDTA and 1 mM DTT. Fractions containing CCS [identified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)] were combined and exchanged via ultrafiltration (Amicon stirred cell with a 30 kDa molecular mass cutoff membrane) into 5 mM Tris (pH 7.5) containing 1 mM DTT. The final purification step used a Resource Q column (6 mL, GE Healthcare) with a 0 to 300 mM NaCl gradient, and fractions containing pure protein (≥90% as judged by SDS−PAGE) were combined.

Expression and Purification of D1-CCS. D1-CCS was expressed and purified as described for the full-length protein except that the final purification step was performed on a Superdex 75 column (16/60 GE Healthcare) in 20 mM Tris (pH 7.5) with 200 mM NaCl and 1 mM DTT. D1-CCS eluted from this column as a single peak.

Expression and Purification of Ccs1. Expression of Ccs1 was achieved as described for CCS except that Zn(II) was omitted from the medium, and Ccs1 was purified using a modified version of a reported procedure.[26] Thawed cell pellets were resuspended in 80 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.0) containing 4 mM EDTA and 4 mM DTT, sonicated, and centrifuged at 35000*g* for 20 min, and the supernatant was diluted 4-fold prior to being loaded onto a SP Sepharose FF column (~40 mL, GE Healthcare). The protein was eluted with a linear NaCl gradient (0 to 500 mM) in 20 mM Mes (pH 6.0) containing 1 mM EDTA and 1 mM DTT. Fractions containing Ccs1 (identified by SDS−PAGE) were combined and exchanged using ultrafiltration (Amicon stirred cell with a 10 kDa molecular mass cutoff membrane) into 20 mM Tris (pH 7.5) with 200 mM NaCl and 1 mM DTT. The final purification step involved a Superdex 200 column (16/60 GE Healthcare) in 20 mM Tris (pH 7.5) with 200 mM NaCl and 1 mM DTT.

D3-CCS and the β-Secretase C-Terminal Domain Peptides. Peptides corresponding to D3 (Gly235−Leu274, GLFQNPKQICSCDGLTIWEERGRPIAGKGRKESAQPPAHL)

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of CCS (D3-CCS) and the cytoplasmic C-terminal domain (CTD, Cys478−Lys501, CQWRCLRCLRQQ_HDDFADDISLLK) of β-secretase (BACE1)[30] were purchased from GeneCust Europe (≥95 and 90% pure, respectively). The solid peptides, stored anaerobically, were dissolved directly in buffer immediately prior to use.

Protein Reduction and Quantification. Proteins (200−800 μM) were reduced by incubation with 10 mM DTT in 20 mM Tris (pH 7.5) in an anaerobic chamber (Belle Technology, ≪2 ppm O_2), typically for 2−3 h. The samples were desalted and exchanged into the required buffer using a PD10 column (GE Healthcare). Thiol quantification using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was routinely used for determining the concentration of D1-CCS (three thiols per monomer), which has little absorbance at 280 nm, and for the assessment of the number of thiols in WT-CCS, C244S/C246S-CCS, C22S/C25S-CCS, and Ccs1. The molar absorption coefficient (ε value) of the thio-(2-nitrobenzoate) product of the reaction of DTNB with thiols (14150 M^{-1} cm^{-1} at 412 nm[32]) was verified using a solution of cysteamine with a known concentration. Ellman's assays were performed anaerobically in 100 mM potassium phosphate and 1 mM EDTA (pH 8.0) containing 500 μM DTNB. The concentrations of WT-CCS, C22S/C25S-CCS, C244S/C246S-CCS, and Ccs1 were routinely determined using calculated ε values at 280 nm (ε_{280}) of 12490 M^{-1} cm^{-1} (for monomeric CCS) and 30940 M^{-1} cm^{-1} (for Ccs1).[33] Concentrations determined from Bradford assays (Coomassie Plus protein assay kit, Thermo Scientific) for WT-CCS, C22S/C25S-CCS, and C244S/C246S-CCS using BSA standards required correction factors of 1.1−1.3 against values obtained using the absorbance at 280 nm. Bradford assays of D1-CCS using BSA standards required a correction factor of 1.5 compared to concentrations determined from Ellman's assays. A set of D1-CCS standards, whose concentrations were determined using DTNB, were also used for Bradford assays of mixtures of apo- and Cu(I)-D1-CCS.

The D3-CCS and BACE1-CTD peptides were quantified using DTNB assuming two and three thiols, respectively. Bradford assays of D3-CCS using BSA standards required a correction factor of 1.7 compared to concentrations determined with DTNB (concentrations obtained using a calculated[33] ε_{280} value of 5500 M^{-1} cm^{-1} were approximately 20% higher). Reduction of the peptide with an immobilized tris(2-carboxyethyl)phosphine gel (Thermo Scientific) overnight did not alter the thiol concentration. The BACE1-CTD peptide concentration determined using a calculated[33] ε_{280} of 5500 M^{-1} cm^{-1} was within 10% of that obtained from thiol quantification.

Atomic Absorption, Circular Dichroism, and Analytical Gel Filtration. Atomic absorption spectroscopy (AAS)[34] and far-UV circular dichroism (CD)[35] spectroscopy were performed as described previously. Analytical gel filtration chromatography was routinely performed on a Superdex 75 column (10/300 GL, GE Healthcare) in 20 mM Mes (pH 6.5) or 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) (pH 7.5) containing 200 mM NaCl at a flow rate of 0.8 mL/min as previously described.[34] Absorbance was measured at 280 nm (240 nm for D1-CCS), and the typical injection volume of samples (10−200 μM) was 150 μL.

Copper(I) Binding and Stoichiometry. A Cu(I) stock solution {50 mM $[Cu(CH_3CN)_4]PF_6$ in 100% acetonitrile} was diluted 50-fold into the appropriate buffer and added to proteins using a gastight syringe (Hamilton). Copper concentrations were determined using either bathocuproine disulfonate (BCS) or bicinchoninic acid (BCA) [chromophoric ligands for Cu(I)], and occasionally by AAS. For measurements using BCS and BCA, ε_{483} and ε_{562} values of 12500 and 7700 M^{-1} cm^{-1} for $[Cu(BCS)_2]^{3-}$ and $[Cu(BCA)_2]^{3-}$, respectively, were used. These were determined by titrating a $[Cu(CH_3CN)_4]$-PF_6 solution (standardized by AAS) into BCS or BCA (40 μM) in 20 mM Mes (pH 6.5) containing 200 mM NaCl.[35−38] To determine the stoichiometry of tight Cu(I) binding, titrations of Cu(I) into fully reduced protein samples (10 μM) in the presence of 50−500 μM BCA were performed in 20 mM Mes (pH 6.5) or 20 mM Hepes (pH 7.5) containing 200 mM NaCl in a septum-sealed gastight cuvette (Hellma). UV−vis spectra (200−800 nm) were recorded on a λ35 spectrophotometer (Perkin-Elmer).

Cu(I) Affinity Determinations. Cu(I) affinities (K_b values) were determined by competition assays with BCS and BCA using an approach described previously.[35,36,38,39] $[Cu(BCS)_2]^{3-}$ has an overall stability constant (β value) of 6.3×10^{19} M^{-2} (at pH ≥8.0),[40] and alterations in the β value with pH were calculated assuming a pK_a of 5.7,[40] as described previously.[35] A β value was estimated for $[Cu(BCA)_2]^{3-}$ from titrations of BCS and BCA with D1-CCS (vide infra). Cu(I) affinity titrations were performed by adding either BCS or BCA (25−100 mM) to a solution of Cu(I)-bound protein (5−20 μM) containing an excess of reduced apo-protein [5−20 μM, with ≤0.5 equiv of Cu(I) in all cases]. Titrations of BCA into WT, C244S/C246S-CCS, and D1-CCS were performed only at pH 6.5 as the concentration of the ligand required for effective competition is too high at pH 7.5. Reduced apo-protein (300−800 μM) was also titrated into a solution of $[Cu(BCS)_2]^{3-}$ or $[Cu(BCA)_2]^{3-}$ (10−15 μM) with an excess of BCS or BCA (10 μM to ∼10 mM), except for WT-CCS and Ccs1 [because of their ability to bind Cu(I) at two distinct sites] and the BACE1-CTD peptide. For D3-CCS, the reduced apo-peptide was titrated into $[Cu(BCA)_2]^{3-}$ at both pH 7.5 and 6.5. In all titrations, equilibration for each point was reached in 10−30 min at room temperature (typically 21 ± 1 °C), except for removal of Cu(I) from C22S/C25S-CCS by BCA, which required much longer times. Therefore, mixtures containing different BCA concentrations and fixed amounts of Cu(I) and C22S/C25S-CCS were incubated for the required amount of time (≤48 h) in the anaerobic chamber. Titrations were performed in either 20 mM Mes (pH 6.5) or 20 mM Hepes (pH 7.5) in the presence of 200 mM NaCl [20 mM Hepes (pH 7.5) with 200 mM KCl for the BACE1-CTD peptide]. To compare the Cu(I) affinities of D1-CCS and HAH1, the reduced apo-proteins (∼300 μM) were individually titrated into $[Cu(BCS)_2]^{3-}$ (15 μM) and excess BCS (120 μM) in 50 mM Hepes (pH 7.5) at an ionic strength of 200 mM (NaCl). All titration data were fit using Origin 7 to a 1:1 Cu(I):protein binding model using eqs 1 and 2 given in ref 35. Fitting of data for titrations (Figure S1 of the Supporting Information) of apo-D1-CCS into $[Cu(BCA)_2]^{3-}$ at pH 7.5 and 6.5 and of BCA into Cu(I)-D1-CCS at pH 6.5 only (vide supra), using the K_b values obtained for D1-CCS from titrations with BCS (Figure S2 of the Supporting Information), allowed β values for $[Cu(BCA)_2]^{3-}$ of $(5.4 \pm 2.7) \times 10^{16}$ and $(5.6 \pm 3.4) \times 10^{16}$ M^{-2} at pH 6.5 and 7.5, respectively, to be estimated. Despite the high Cu(I) affinity of D1-CCS, which limits competition with BCA for Cu(I), particularly at pH 7.5, these values are in good agreement with those reported previously at pH 7.0 (5×10^{16} to 2.6×10^{17} M^{-2}) using proteins with a range of affinities, in some cases higher than that of D1-CCS,[36,37] and 5.4×10^{16} M^{-2} was used as the β value for $[Cu(BCA)_2]^{3-}$ in this study.

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Cu(I) Partitioning and Exchange Assays. $[Cu(BCA)_2]^{3-}$ [1:2.5 Cu(I):BCA] was added to mixtures of reduced D1-CCS with either reduced WT-CCS, C22S/C25S-CCS, or C244S/C246S-CCS (100−200 μM each) in 20 mM Mes (pH 6.5) containing 150 mM NaCl (total volume of 0.5 mL). The mixtures were incubated for up to 4 h and loaded onto a HiTrap Q HP anion exchange column (1 mL, GE Healthcare) pre-equilibrated in the same buffer (all in the anaerobic chamber). D1-CCS did not bind to the column, while WT-CCS, C22S/C25S-CCS, and C244S/C246S-CCS did and were eluted with buffer and 300 mM NaCl (2.0 mL fractions). The partitioning of Cu(I) {also supplied as $[Cu(BCA)_2]^{3-}$} between reduced apo-D1-CCS and reduced apo-D3-CCS (75 μM each) was performed anaerobically in 20 mM Mes (pH 6.5) (total volume of 0.5 mL). D3-CCS did not bind to the HiTrap Q column under these conditions, while D1-CCS was eluted with buffer and 150 mM NaCl (2.0 mL fractions). The protein concentrations in the fractions were determined using Bradford assays and for D1-CCS only checked using thiol quantification, and copper concentrations were measured with BCS and AAS. To investigate Cu(I) exchange, D1-CCS and C244S/C246S-CCS and also D1-CCS and C22S/C25S-CCS, with Cu(I) bound to one of the proteins and the apo-partner fully reduced, were mixed in 20 mM Mes (pH 6.5) containing 150 mM NaCl (total volume of 0.5 mL). Mixtures were incubated for up to 48 h anaerobically. The separation of proteins and the quantification of protein and copper in fractions were performed as described for the Cu(I) partitioning experiments (vide supra). The exchange equilibrium constant (K_{ex} value) for the transfer of copper (copper partitioning is expressed in the same way) from D1-CCS to CCS (either WT-CCS, C22S/C25S-CCS, C244S/C246S-CCS, or D3-CCS) was determined using eq 1:

$$K_{ex} = \frac{[\text{apo-D1-CCS}][\text{Cu(I)-CCS}]}{[\text{Cu(I)-D1-CCS}][\text{apo-CCS}]} \quad (1)$$

in which [apo-D1-CCS] and [Cu(I)-D1-CCS] represent the concentrations of apo- and Cu(I)-D1-CCS, respectively, and [Cu(I)-CCS] and [apo-CCS] the concentrations of the Cu(I) and apo forms of the partner protein (WT-CCS, C22S/C25S-CCS, C244S/C246S-CCS, or D3-CCS), respectively. In the partitioning experiments, the majority of Cu(I) associates with D1 and relatively low levels of Cu(I) bound to D3 (vide infra). This results in very small amounts of residual $[Cu(BCA)_2]^{3-}$, which should not interfere with the analysis of the data.

Exchange of Cu(I) between D1-CCS and HAH1 was performed by mixing Cu(I)-loaded protein with the reduced apo form of the partner protein in 20 mM Hepes (pH 7.5) (total volume of 1.0 mL). Mixtures were incubated for <5 min prior to being loaded onto a HiTrap Q HP column pre-equilibrated in the same buffer. Buffer with 100 and 200 mM NaCl was used to elute HAH1 and D1-CCS [apo and Cu(I) forms in both cases], respectively (2.0 mL fractions). The protein content and copper content of fractions were determined by thiol quantification and AAS, respectively, with K_{ex} calculated using eq 2:

$$K_{ex} = \frac{[\text{apo-D1-CCS}][\text{Cu(I)-HAH1}]}{[\text{Cu(I)-D1-CCS}][\text{apo-HAH1}]} \quad (2)$$

RESULTS AND DISCUSSION

Protein Characterization. WT-CCS, C22S/C25S-CCS, and C244S/C246S-CCS were all isolated with 0.8 ± 0.2 equiv of zinc per monomer (protein concentrations measured using the ε_{280} value) and very little copper. D1-CCS and Ccs1 were purified with negligible copper and zinc. The molecular masses of all purified proteins are within 4 Da of expected values (Table S2 of the Supporting Information). For reduced apo-proteins, the number of thiols ranged from 4.5 to 5.6, from 2.4 to 3.3, and from 3.5 to 4.6 per monomer for WT-CCS, C22S/C25S-CCS, and C244S/C246S-CCS, respectively (4.5−5.0 for Ccs1), based on protein concentrations determined from the ε_{280} value. Using the Zn(II) concentrations, and assuming one Zn(II) per monomer, gave 5.6−6.8, 3.3−4.6, and 4.4−5.8 free thiols per Zn(II) ion, respectively. CCS has nine Cys residues (three in D1, four in D2, and two in D3), while Ccs1 has four Cys residues in D1 (two involved in a disulfide), one in D2, and two in D3. The number of thiols we have determined for WT-CCS is consistent with the disulfide corresponding to the essential disulfide in SOD1 being present in D2.[22] Far-UV CD spectra (Figure 1B) show that the Cys to Ser mutations in the CXXC and CXC motifs of D1 and D3, respectively, do not significantly perturb the secondary structure of the protein (alterations were not anticipated for C244S/C246S-CCS as D3 is largely unstructured). The far-UV CD spectrum of Ccs1 resembles that of CCS (Figure 1B), while the spectrum of D1-CCS (Figure 1B) is similar to those previously reported for HAH1 and related domains.[36,41] Analytical gel filtration chromatography demonstrates that, in the absence of Cu(I), WT-CCS and the Cys to Ser variants are all dimers, and D1-CCS is a monomer (Figure 1C and Figures S3A,D,F and S4A and Table S3 of the Supporting Information). D1-CCS and C244S/C246S-CCS remain monomeric and dimeric, respectively, upon addition of 1 equiv of Cu(I), while mixtures of the dimer and tetramer are found for WT-CCS and C22S/C25S-CCS in the presence of Cu(I) (Figures S3C,G and S4B−D and Table S3 of the Supporting Information).[24] The WT-CCS tetramer binds two Cu(I) ions per monomer, while only a single Cu(I) ion is bound per monomer in tetrameric C22S/C25S-CCS (Figure 2). The amount of tetrameric C22S/C25S-CCS

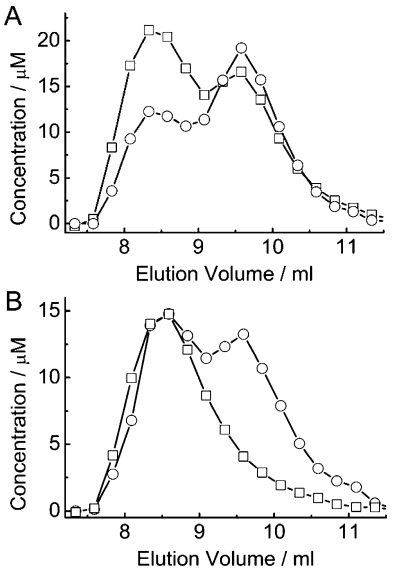

Figure 2. Copper (□) and protein (○) content of the tetrameric (8.5 mL elution volume) and dimeric (9.6 mL elution volume) forms of (A) WT-CCS [200 μM Cu(I)-protein] and (B) C22S/C25S-CCS [200 μM protein with 100 μM Cu(I)] separated by analytical gel filtration chromatography in 20 mM Mes (pH 6.5) and 200 mM NaCl.

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and WT-CCS increases upon increasing the number of Cu(I) equivalents to 1 (Figure S4B–D of the Supporting Information) and 2 (data not shown), respectively. A considerable amount of dimer is still present for C22S/C25S-CCS loaded with 1 equiv of Cu(I) at low micromolar protein concentrations (Figure S4C of the Supporting Information), but very little dimer is observed when the protein concentration is 10 times higher (Figure S4D of the Supporting Information). The dissociation constant for the tetramer would therefore appear to be in the micromolar range and is potentially physiologically relevant.[2,24] Apo-Ccs1 is monomeric (Figure 1C), and a mixture of the monomer and dimer is present upon addition of Cu(I).[3,23]

Cu(I) Binding Stoichiometries and Cu(I) Affinities. Cu(I) binding stoichiometries were determined by titrating Cu(I) into the apo-proteins in the presence of BCA. WT-CCS, C244S/C246S-CCS, and D1-CCS all bind 1 equiv of Cu(I) tightly (compared to BCA) per monomer at both pH 6.5 and 7.5 (Figure 3A–C and Figure S5A–C of the Supporting Information), presumably via the CXXC motif of D1. Cu(I) binds to a lower-affinity site in WT-CCS, similar to that in C22S/C25S-CCS (Figure 3A,D and Figure S5A,D of the Supporting Information), most likely the CXC motif of D3. Higher concentrations of BCA (500 μM) are required to compete with this site for Cu(I) at pH 7.5 versus that at pH 6.5, indicating increased affinity. Experiments at pH 7.5 with lower concentrations of BCA (150 μM) give Cu(I) binding stoichiometries of 1:1 per monomer of C22S/C25S-CCS (Figure 4), as also found by gel filtration (Figure 2B). These data and Cu(I) affinity and transfer results (vide infra) are consistent with the tetrameric form of CCS consisting of D2-linked dimers connected by CXC motifs that each tightly bind a single Cu(I) ion, forming two Cu_2 clusters[42] at two D3–D3 interfaces. Such an arrangement is probably present at both lower [0.5 Cu(I) per D3 (Figure 2B and Figures S3G and S4B of the Supporting Information)] and higher [1.0 Cu(I) per D3 (Figure S4C,D of the Supporting Information)] copper occupancies, with the amount depending on protein concentration (vide supra). Dissociation of the tetramer into dimers therefore most likely involves the disruption of two D3 interfaces, which is expected to be a relatively slow process and may be responsible for the slow removal of Cu(I) from D3 of CCS by BCA (Figure 5) (the coexistence of dimeric and tetrameric forms probably results in the biphasic nature of this process). For Ccs1, Cu(I) binding data are also indicative of the presence of two Cu(I) sites with significantly different affinities (Figure S6 of the Supporting Information).

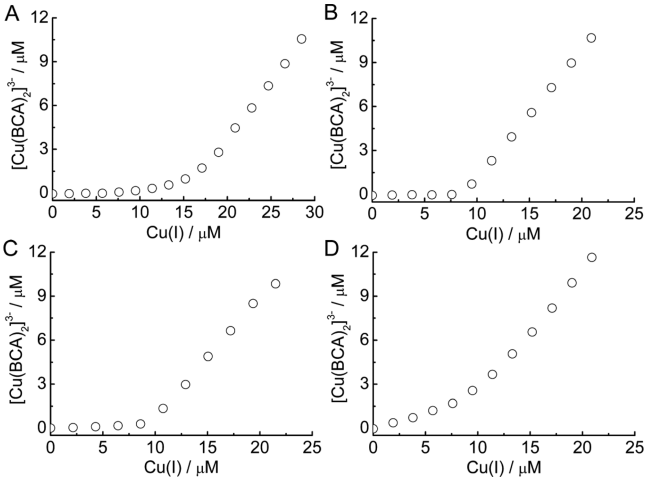

Figure 3. Titrations of Cu(I) into reduced apo-WT-CCS (A), apo-C244S/C246S-CCS (B), apo-D1-CCS (C), and apo-C22S/C25S-CCS (D) (all at 10 μM) in the presence of 50 μM BCA in 20 mM Mes (pH 6.5) and 200 mM NaCl.

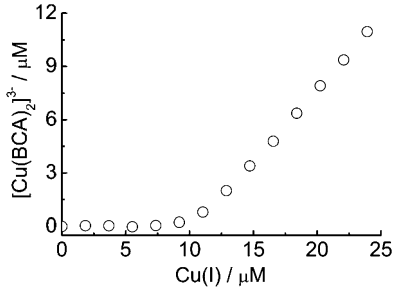

Figure 4. Titrations of Cu(I) into reduced apo-C22S/C25S-CCS (10 μM) in the presence of 150 μM BCA in 20 mM Mes (pH 7.5) and 200 mM NaCl.

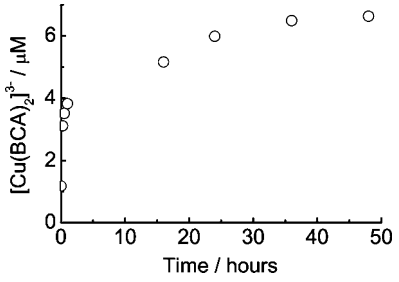

Figure 5. Time course of removal of Cu(I) from C22S/C25S-CCS [20 μM Cu(I)-protein] by BCA (500 μM) in 20 mM Mes (pH 6.5) containing 200 mM NaCl. The final point gives a K_b of 3.9×10^{15} M^{-1}.

The copper affinities (K_b values) of D1 and D3 were determined from competition titrations with BCS and BCA at pH 7.5 and 6.5. Experiments at pH 7.5 provide Cu(I) affinities that can readily be compared with literature values for related systems (typically measured at pH 7–8), while at pH 6.5, the same ligand (BCA) can be used for all proteins. The Cu(I) affinity measured using BCS for the first equivalent of Cu(I) bound to D1 of WT-CCS [using a Cu(I):CCS ratio of $\leq$0.5 under which conditions negligible tetramer is present (Figure S3B of the Supporting Information)] at pH 7.5 is $(5.5 \pm 0.6) \times 10^{17}$ M^{-1}, and almost identical values are found for C244S/

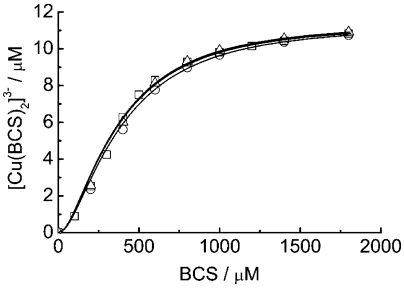

Figure 6. Titrations of BCS into Cu(I)-protein (10 μM) and apo-protein (10 μM) for WT-CCS (○), C244S/C246S-CCS (□), and D1-CCS (△) in 20 mM Hepes (pH 7.5) and 200 mM NaCl. The lines show fits of the data to eq 2 from ref 35 giving K_b values of $(5.8 \pm 0.2) \times 10^{17}$, $(5.2 \pm 0.3) \times 10^{17}$, and $(5.1 \pm 0.2) \times 10^{17}$ M^{-1}, respectively.

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Table 1. Cu(I) Affinities (K_b Values) of CCS and Ccs1

protein	pH	K_b (BCA) (M^{-1})	K_b (BCS) (M^{-1})
WT-CCS[a]	7.5	–	$(5.5 \pm 0.6) \times 10^{17}$
	6.5	$(1.1 \pm 0.6) \times 10^{17}$	$(4.4 \pm 1.0) \times 10^{16}$
C244S/C246S-CCS[b]	7.5	$(1.1 \pm 0.6) \times 10^{18}$	$(4.6 \pm 1.0) \times 10^{17}$
	6.5	$(0.9 \pm 0.5) \times 10^{17}$	$(4.6 \pm 2.5) \times 10^{16}$
D1-CCS[c]	7.5	–	$(5.5 \pm 3.5) \times 10^{17}$
	6.5	–	$(5.6 \pm 2.6) \times 10^{16}$
C22S/C25S-CCS[d]	7.5	$(2.7 \pm 1.4) \times 10^{16}$	–
	6.5	$(2.9 \pm 1.7) \times 10^{15}$	–
D3-CCS[e]	7.5	$(6.4 \pm 0.3) \times 10^{16}$	–
	6.5	$(4.2 \pm 1.0) \times 10^{15}$	–
Ccs1[f]	7.5	–	$(2.4 \pm 0.5) \times 10^{17}$
	6.5	–	$(2.7 \pm 1.0) \times 10^{16}$

[a]Data for the first equivalent of Cu(I) from titrations of BCA and BCS into Cu(I)-protein only [titrations using reduced apo-WT-CCS were not performed because of its ability to bind two Cu(I) ions]. The Cu(I) affinity of the first equivalent of copper, bound at the CXXC motif of D1, is too high at pH 7.5 to be measured by titrating BCA into Cu(I)-protein. [b]The K_b value at pH 7.5 using BCA was obtained only by titrating apo-C244S/C246S-CCS into $[Cu(BCA)_2]^{3-}$. [c]D1-CCS was used for the estimation of the β value for $[Cu(BCA)_2]^{3-}$, and therefore, K_b values using this ligand are not included. [d]Data from competition experiments with BCS are not included because titrations of apo-C22S/C25S-CCS into $[Cu(BCS)_2]^{3-}$ level off well before all of the copper has been removed from the complex, possibly because of the formation of a BCS–Cu(I)–C22S/C25S-CCS ternary complex. Similar behavior is not observed in titrations of apo-C22S/C25S-CCS into $[Cu(BCA)_2]^{3-}$, and the affinity obtained is very similar to that from the titration of BCA into Cu(I)-C22S/C25S-CCS. [e]From titrations of the apo-peptide into $[Cu(BCA)_2]^{3-}$ only. [f]Data for the first equivalent of Cu(I) from titrations of BCS into Cu(I)-protein only.

C246S-CCS and D1-CCS (Figure 6, Table 1, and Figures S2A,C and S7A,C of the Supporting Information). The Cu(I) affinities for these three proteins are also the same at pH 6.5 (Table 1 and Figures S2B,D and S7B,D of the Supporting Information), albeit the values are approximately 10-fold weaker than at pH 7.5 because of protonation of a Cys ligand.[35] There are minor differences (2–3-fold) between the Cu(I) affinities of WT-CCS and C244S/C246S-CCS determined using BCS and those measured with BCA at both pH values (Table 1), which are within the error of our estimation of the β value for $[Cu(BCA)_2]^{3-}$ [$(5.4 \pm 2.7) \times 10^{16}$ M^{-2}]. The first equivalent of Cu(I) bound to Ccs1 from *S. cerevisiae* has a Cu(I) affinity of $(2.4 \pm 0.5) \times 10^{17}$ M^{-1} at pH 7.5 [experiment performed in the presence of 0.5 equiv of Cu(I) (Figure 8A)], which decreases ~10-fold at pH 6.5 (Table 1) and presumably is also for the CXXC site in D1. The Cu(I) affinities of D1 of CCS and Ccs1, and also of the CTD of BACE1 [$(2.3 \pm 0.5) \times 10^{17}$ M^{-1} at pH 7.5 (Figure 8B)], are all in the same range as values reported for the metallochaperones HAH1 (vide infra),[35,39] Atx1 from *S. cerevisiae* and *Synechocystis* PCC 6803,[36,39,40] and CopZ from *Bacillus subtilis*,[43] proteins that all contain a CXXC motif.

The lower Cu(I) affinity of BCA allows the K_b value of the CXC site in D3 to be measured in C22S/C25S-CCS (Figure 7B,D) and also in the D3-CCS peptide (Figure 8C). The apparent Cu(I) affinity of this site is more than 1 order of magnitude weaker than that of the CXXC motif of D1 at both pH 7.5 and 6.5 (Table 1). Considering that Cu(I) bound by the CXC motif can be present as a Cu_2 cluster in tetrameric CCS, the measured K_b has a contribution from tetramer formation,[36] and the absolute Cu(I) affinity of the CXC motif in the dimer is expected to be weaker.

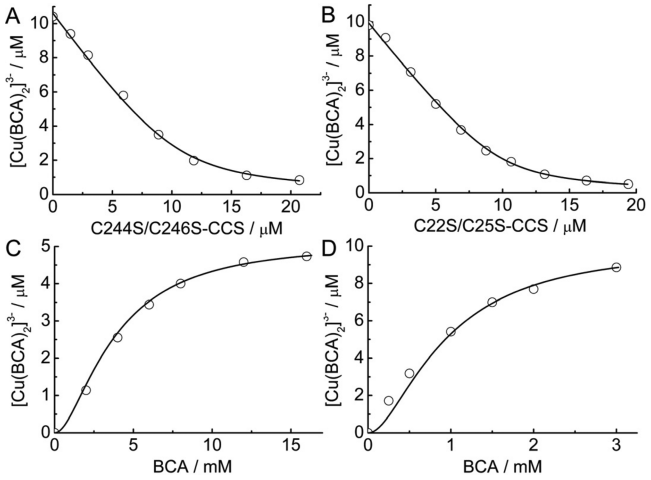

Figure 7. Titrations of (A) apo-C244S/C246S-CCS and (B) apo-C22S/C25S-CCS into $[Cu(BCA)_2]^{3-}$ (10 μM) in the presence of 1180 and 150 μM BCA, respectively. Titrations of BCA into (C) Cu(I)-C244S/C246S-CCS (5 μM) and apo-C244S/C246S-CCS (5 μM) and (D) Cu(I)-C22S/C25S-CCS (10 μM) and apo-C22S/C25S-CCS (10 μM). All titrations were performed in 20 mM Mes (pH 6.5) and 200 mM NaCl. The lines show fits of the data to eqs 1 (A and B) and 2 (C and D) from ref 35, giving K_b values of $(8.8 \pm 0.6) \times 10^{16}$ (A), $(2.2 \pm 0.6) \times 10^{15}$ (B), $(1.0 \pm 0.1) \times 10^{17}$ (C), and $(3.0 \pm 0.4) \times 10^{15}$ M^{-1} (D).

Partitioning and Exchange of Cu(I) between Domains 1 and 3. The removal of Cu(I) (1 equiv) from C22S/C25S-CCS by BCA (Figure 5) is biphasic (vide supra) and is significantly slower than for all other proteins studied. Slow removal of Cu(I) is observed for WT-CCS loaded with >1 equiv of Cu(I), and copper cluster formation by CCS must be responsible for this behavior. To investigate the partitioning of copper between D1 and D3 and also to verify the relative Cu(I) affinities obtained from the competition titrations with chromophoric ligands, Cu(I) was added as $[Cu(BCA)_2]^{3-}$ to D1-CCS and either WT-CCS, C22S/C25S-CCS, C244S/C246S-CCS, or D3-CCS. The mixtures were separated, and copper partitioning, expressed as K_{ex}, was assessed (Table 2 and Tables S4–S7 of the Supporting Information). In all cases, the reactions are largely complete within minutes, indicating that equilibration of Cu(I) from $[Cu(BCA)_2]^{3-}$ between domains is relatively fast (occurs within the dead time of the experiment, which is approximately 2 min). Direct Cu(I) exchange has also been studied and is relatively fast for transfers involving D1-CCS and C244S/C246S-CCS and from D1-CCS to C22S/C25S-CCS (Table 3 and Tables S8 and S9 of the Supporting Information). The transfer from C22S/C25S-CCS to D1-CCS is much slower, requiring up to 48 h for equilibration (Table 3 and Table S9 of the Supporting Information), presumably because of slow dissociation of the cluster involving the CXC site of D3. In all cases {partitioning from $[Cu(BCA)_2]^{3-}$ and Cu(I) exchange}, the K_{ex} values obtained experimentally are in good agreement with those calculated on the basis of Cu(I) affinities (Tables 2 and 3), and the low BCA concentration relative to the protein concentrations present in the partitioning experiments therefore has no influence. These data are consistent with the CXXC site having similar Cu(I) affinities in WT-CCS, C244S/C246S-CCS, and D1-CCS. More importantly, the data for the partitioning and transfer of copper between D1 and D3 confirm that the CXXC motif of

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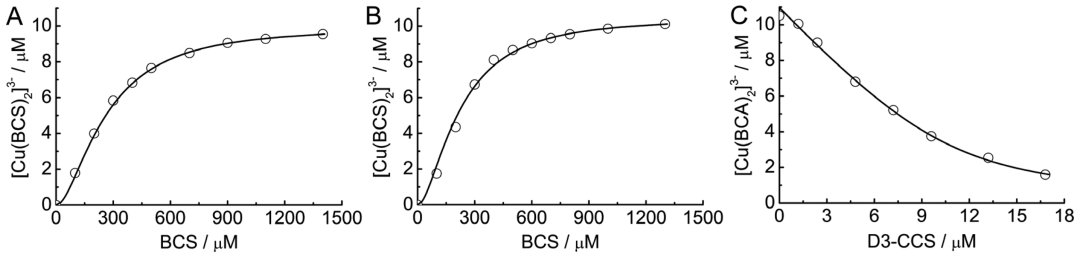

Figure 8. Titrations of BCS into (A) Cu(I)-Ccs1 (10 μM) and apo-Ccs1 (10 μM), and (B) Cu(I)-BACE1-CTD (10 μM) and apo-BACE1-CTD (10 μM) at pH 7.5. The titration of apo-D3-CCS into $[Cu(BCA)_2]^{3-}$ (10 μM) and 330 μM BCA at pH 6.5 is shown in panel C. The lines show fits of the data to eqs 2 (A and B) and 1 (C) from ref 35, giving K_b values of $(2.5 \pm 0.1) \times 10^{17}$, $(1.8 \pm 0.1) \times 10^{17}$, and $(5.0 \pm 0.3) \times 10^{15}$ M^{-1}, respectively.

Table 2. K_{ex} Values for Partitioning of Cu(I) from $[Cu(BCA)_2]^{3-}$ between a Mixture of Two Apo-Proteins[a]

protein mixture	incubation time	K_{ex}	K_{ex}^{calc} [b]
D1-CCS and WT-CCS	min[c]	1.7	2.0
	1 h	1.7	
	4 h	1.8	
D1-CCS and C244S/C246S-CCS	min[c]	2.1	1.6
	1 h	1.8	
D1-CCS and C22S/C25S-CCS	min[c]	0.09	0.05
	1 h	0.13	
	4 h	0.11	
D1-CCS and D3-CCS	min[c]	0.09	0.08

[a]Performed in 20 mM Mes (pH 6.5) containing 150 mM NaCl. [b]Calculated from the K_b values of the partner proteins obtained with BCA (except for that of D1-CCS, which was determined with BCS only) using the relationship $K_{ex}^{calc} = K_b$(WT-CCS, C244S/C246S-CCS, C22S/C25S-CCS, or D3-CCS)/K_b(D1-CCS). [c]Proteins were separated in approximately 2 min.

Table 3. K_{ex} Values for Exchange of Cu(I) between Proteins[a]

protein mixture	incubation time	K_{ex}	K_{ex}^{calc} [b]
Cu(I)-D1-CCS and apo-C244S/C246S-CCS	min[c]	1.5	1.6
	1 h	1.8	
apo-D1-CCS and Cu(I)-C244S/C246S-CCS	min[c]	1.4	
	1 h	1.4	
Cu(I)-D1-CCS and apo-C22S/C25S-CCS	min[c]	0.03	0.05
	1 h	0.06	
	4 h	0.08	
apo-D1-CCS and Cu(I)-C22S/C25S-CCS	min[c]	3.4	
	1 h	1.4	
	48 h	0.16	
apo-D1-CCS and Cu(I)-HAH1	min[c]	0.9	1.7
Cu(I)-D1-CCS and apo-HAH1	min[c]	1.1	

[a]Performed in 20 mM Mes (pH 6.5) containing 150 mM NaCl except for exchange experiments between D1-CCS and HAH1, which were performed in 20 mM Hepes (pH 7.5). [b]Calculated from the K_b values of the partner proteins obtained with BCA (except for that of D1-CCS, which was determined with BCS only) using the relationship $K_{ex}^{calc} = K_b$(WT-CCS, C244S/C246S-CCS, C22S/C25S-CCS, or D3-CCS)/K_b(D1-CCS) or $K_{ex}^{calc} = K_b$(HAH1)/K_b(D1-CCS) (K_b values obtained with BCS). [c]Proteins were separated in approximately 2 min.

D1 has a Cu(I) affinity that is at least 10-fold higher than that of the CXC site of D3.

Physiological Significance of the Measured Cu(I) Affinities. The CXXC motif in D1 of the copper metallochaperone for Cu,Zn-superoxide dismutase is not conserved in all organisms (vide infra), which has led to the suggestion that this domain may be important only for interactions and not for the binding of copper.[44] However, the higher Cu(I) affinity of D1 compared to that of D3, along with studies showing that the CXXC motif is essential for SOD1 activation in vivo,[27] demonstrates that in humans D1 of CCS must play a direct role in the trafficking of copper to SOD1. Furthermore, we show that Cu(I) from $[Cu(BCA)_2]^{3-}$, a complex previously used as a copper transfer partner mimic for a copper metallochaperone,[41] partitions rapidly between D1 and D3 binding predominantly to D1 [data obtained using D1 and D3 sites in separate proteins (Table 2) that also show preferential acquisition of Cu(I) by D1 in direct exchange experiments (Table 3)]. Domain 1 is therefore expected to play a role in acquiring Cu(I) and, considering the tightly buffered nature of cellular copper,[2,29,45] will be the primary location for Cu(I) on CCS. Only a small proportion of Cu(I) bound to D1 will transfer to D3, and the D1 site is therefore expected to remain metalated until the metallochaperone docks with the target. The mechanism of metallochaperone-mediated Cu,Zn-superoxide dismutase activation is not completely understood. Most information is available for *S. cerevisiae* Sod1 activation, and the crystal structure of the complex with Ccs1 in the absence of copper has been determined (Figure 1A).[19] In this complex, the Cys residues of D3 are ideally placed for shuttling Cu(I) to the copper site of Sod1, whereas the CXXC motif of D1 is ~35 Å distant. The location of D3 and its mobility (a short partially structured domain[19] not observed in the structure of Ccs1 alone[18]) led to the proposal that Cu(I) bound to D1 could be shuttled to Sod1 via D3 in the complex.[19] If a similar complex forms between the human proteins, and the Cu(I) affinities of CCS are not altered in the heterodimeric complex with SOD1 compared to the homodimer, the transfer of Cu(I) from D1 to D3 will be thermodynamically unfavorable. The unconstrained flexibility of D3 of CCS[46] along with the coordinatively unsaturated nature of two-coordinate Cu(I) bound by a CXXC site that makes the metal susceptible to attack from exogenous ligands,[6,7,20,34,47−49] such as a Cys residue from D3, will facilitate Cu(I) transfer via ligand exchange. The Cu(I) affinity of SOD1 is ~10-fold tighter than that of CCS loaded with 1 equiv of Cu(I),[29] which we show is primarily bound to D1, and therefore >100-fold tighter than the Cu(I) affinity of D3. The ability of the structurally flexible D3 to access both the CXXC motif of D1 and the relatively buried active site of SOD1 would appear to be more important than its affinity for Cu(I). The CXC motif of D3 also plays a role in disulfide bond formation in the target between Cys57 and Cys146, which requires the metallochaperone to be copper-loaded, and Cu(I)

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bound by D3 will come into its proximity with these two Cys residues during activation.[10] Disulfide formation probably involves an intermediate with an intermolecular disulfide (between Cys229 of Ccs1 and Cys57 of Sod1, which is present in the structure of the complex[19]) and requires oxygen.[9,10] During this process, copper oxidation may facilitate transfer of the metal to the tetragonal, all-His ligand, copper site of the target, which will favor Cu(II) binding compared to the all-thiolate, and lower-coordination number, sites in the metallochaperone.

The CCS from *Drosophila melanogaster* provides a rare example of this copper metallochaperone without the CXXC motif,[28] and therefore, the high-affinity Cu(I) site of D1 is absent. The D1s of the *Schizosaccharomyces pombe*[50] and *Anopheles gambiae*[28] proteins have only one of these Cys residues, although D1 is still required for target activation at low available copper concentrations in *S. pombe*.[50] In *S. cerevisiae*, D1 of Ccs1 has been shown to be required for Sod1 activation under copper-limiting conditions.[3] Interestingly, the C17S/C20S-Ccs1 variant, in which the Cys residues of the CXXC motif have been mutated, is still capable of activating Sod1, although the effect of copper availability has not been investigated.[28] An alternative mechanism for the activation of Cu,Zn-superoxide dismutase that does not involve the copper metallochaperone exists[44,51−53] but utilizes glutathione,[51] with disulfide bond formation occurring in the absence of copper and oxygen. The disulfide bond in *S. cerevisiae* Sod1 can be formed by only Ccs1, because of the presence of Pro144.[51,53] It would appear that for *S. cerevisiae* a role for D1 in acquiring copper under limiting conditions, consistent with the high Cu(I) affinity we have determined, is potentially more important than in organisms that can activate the enzyme via the alternative route (activation via CCS is the more efficient mechanism in humans[44]). The potentially detrimental effect, under copper-limiting conditions, of the absence of the CXXC motif in *Drosophila* may be minimized by the stability of metalated SOD1 and slowed cell turnover.[28]

Copper clusters bound by the CXC motif of D3 form upon binding of Cu(I) to CCS in vitro.[24,42] Both Cu_2 and Cu_4 clusters involving dimeric and tetrameric forms of the protein have been reported, with the larger cluster requiring additional thiolate ligands, possibly provided by Cys residues in D2.[54] We show that the CXC site of D3 binds 1 equiv of Cu(I) tightly, indicating that the CCS tetramer is linked by two Cu_2 clusters both bound by one CXC motif from each dimer. Copper is released very slowly from this form of the protein (Figure 5 and Table 3), which could hinder cellular copper trafficking. D1 may therefore play a role in limiting binding of Cu(I) to D3 of CCS, and undesirable cluster formation, until a complex has been formed with SOD1 (if copper cluster and tetramer formation is sufficiently slow in vivo, D1 may not be required to prevent the formation of this species). Furthermore, transient binding of Cu(I) to the CXC motif of D3 of CCS in the heterocomplex with SOD1 will also preclude cluster formation.

A pool of copper exists in the eukaryotic cytosol,[2,29,45] and the matched Cu(I) affinities of CCS (D1) and HAH1, and of Ccs1 (D1) and Atx1 [although the K_b of Ccs1 determined here at pH 7.5 indicates a slightly weaker affinity for Cu(I) compared to reported values for Atx1 of 5.0×10^{17} and 1.6×10^{18} M^{-1} at pH 7.0 and 8.0, respectively[39,40]], along with similar protein levels (quantified in *S. cerevisiae*[2,55]) highlight that thermodynamically these metallochaperones can compete for available copper. It has been suggested that CCS and HAH1 may obtain Cu(I) from the same location in the cytosol, possibly the high-affinity copper importer CTR1,[56] although both Atx1 and Ccs1 can acquire copper via other transporters,[57] and copper can be transferred from Ctr1 to Atx1 (*S. cerevisiae*) in vitro.[40] The surface properties, particularly in the vicinity of the CXXC motifs, differ between these two copper metallochaperones, and in vivo complementation experiments in *S. cerevisiae* show that D1 of Ccs1 and Atx1 are not interchangeable,[3] a clear demonstration of the importance of protein interactions for copper trafficking pathways.[49] The surface features of these two metallochaperones may be more important for copper delivery than acquisition, however, as they transfer copper to very different targets; HAH1 interacts with copper-transporting P-type ATPases (ATP7A and ATP7B), whereas CCS delivers it directly to the copper-requiring enzyme. We have found that copper readily transfers between D1-CCS and HAH1 with a K_{ex} of 1.1 (Table 3 and Table S10 of the Supporting Information), consistent with their K_b values [$(5.4 \pm 0.2) \times 10^{17}$ and $(9.3 \pm 0.1) \times 10^{17}$ M^{-1}, respectively, at pH 7.5]. Cross-talk could therefore occur between the two cytosolic copper trafficking pathways via the metallochaperones, with the availability of, and copper uptake by, SOD1 and the ATPases being factors that will favor passage of the metal down either route. In support of this idea, it has recently been shown that in hypoxic macrophages, the flow of copper to the secretory pathway via ATP7A increases, accompanied by a lowered level of expression of CCS and a decrease in SOD1 (and cytochrome *c* oxidase) activity.[58] Unlike Cu(I) bound to D1 of CCS, metal coordinated by the D3 site could be readily lost to HAH1 and other Cu(I) sites and ligands in the cytosol, unless it is trapped in the kinetically stable tetrameric form.

Another potential cytosolic binding site for Cu(I) is found in BACE1, which catalyzes the first step in the processing of the amyloid precursor protein to yield Aβ, a major constituent of senile plaques in the brains of AD patients. BACE1 is able to interact with D1 of CCS and HAH1 via the CTD that coordinates Cu(I), prompting suggestions that binding of Cu(I) to BACE1 may modulate the production of Aβ.[30] The Cu(I) affinity of the BACE1-CTD is somewhat weaker than those of D1-CCS and HAH1, which will limit competition with the metallochaperones for cytosolic copper under equilibrium conditions.

■ CONCLUSIONS

We have found that the Cu(I) affinity of the CXXC motif in D1 of human CCS is at least 10-fold higher than that of the CXC motif in D3, which also seems to be the case for *S. cerevisiae* Ccs1. This is consistent with D1 being involved in the acquisition of copper, particularly under limiting conditions. The high Cu(I) affinity of D1 and transient metalation of D3 could help prevent Cu(I) cluster formation involving D3 in vivo. Matched Cu(I) affinities and facile Cu(I) exchange involving CCS and HAH1 demonstrate that cross-talk between cytosolic copper trafficking pathways could occur via the metallochaperones.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables showing primers (Table S1), mass spectrometry data (Table S2), analytical gel filtration data (Table S3), and Cu(I) partitioning and exchange data (Tables S4−S10) and figures showing Cu(I) affinity titrations (Figures S1, S2, and S7), analytical gel filtration chromatograms (Figures S3 and S4), and Cu(I) stoichiometry titrations (Figures S5 and S6). This

material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

SOD1, human Cu,Zn-superoxide dismutase; Sod1, *S. cerevisiae* Cu,Zn-superoxide dismutase; AD, Alzheimer's disease; CCS, human copper metallochaperone for Cu,Zn-superoxide dismutase; Ccs1, *S. cerevisiae* copper metallochaperone for Cu,Zn-superoxide dismutase; D1, domain 1; D2, domain 2; D3, domain 3; WT-CCS, wild-type CCS; C22S/C25S-CCS, C22S/C25S double mutant of CCS; C244S/C246S-CCS, C244S/C246S double mutant of CCS; D1-CCS, D1 construct of CCS; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Mes, 2-(*N*-morpholino)ethanesulfonic acid; D3-CCS, D3 of CCS; BACE1-CTD, cytoplasmic C-terminal domain of β-secretase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent); ε, molar absorption coefficient; AAS, atomic absorption spectroscopy; CD, circular dichroism; Hepes, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; BCA, bicinchoninic acid; BCS, bathocuproine disulfonate; K_b, Cu(I) affinity; β, overall stability constant; K_{ex}, copper exchange equilibrium constant.

REFERENCES

(1) Culotta, V. C., Klomp, L. W. J., Strain, J., Casareno, R. L. B., Krems, B., and Gitlin, J. D. (1997) The copper chaperone for superoxide dismutase. *J. Biol. Chem. 272*, 23469–23472.

(2) Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and O'Halloran, T. V. (1999) Undetectable intracellular free copper: The requirement of a copper chaperone for superoxide dismutase. *Science 284*, 805–808.

(3) Schmidt, P. J., Rae, T. D., Pufahl, R. A., Hamma, T., Strain, J., O'Halloran, T. V., and Culotta, V. C. (1999) Multiple protein domains contribute to the action of the copper chaperone for superoxide dismutase. *J. Biol. Chem. 274*, 23719–23725.

(4) Wong, P. C., Waggoner, D., Subramaniam, J. R., Tessarollo, L., Bartnikas, T. B., Culotta, V. C., Price, D. L., Rothstein, J., and Gitlin, J. D. (2000) Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A. 97*, 2886–2891.

(5) Rae, T. D., Torres, A. S., Pufahl, R. A., and O'Halloran, T. V. (2001) Mechanism of Cu,Zn-superoxide dismutase activation by the human copper metallochaperone hCCS. *J. Biol. Chem. 276*, 5166–50176.

(6) Rosenzweig, A. C. (2001) Copper delivery by metallochaperone proteins. *Acc. Chem. Res. 34*, 119–128.

(7) Huffman, D. L., and O'Halloran, T. V. (2001) Function, structure, and mechanism of intracellular copper trafficking proteins. *Annu. Rev. Biochem. 70*, 677–701.

(8) Culotta, V. C., Yang, M., and O'Halloran, T. V. (2006) Activation of superoxide dismutases: Putting the metal to the pedal. *Biochim. Biophys. Acta 1763*, 747–758.

(9) Brown, N. M., Torres, A. S., Doan, P. E., and O'Halloran, T. V. (2004) Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A. 101*, 5518–5523.

(10) Furukawa, Y., Torres, S. A., and O'Halloran, T. V. (2004) Oxygen-induced maturation of SOD1: A key role for disulfide formation by the copper chaperone CCS. *EMBO J. 23*, 2872–2881.

(11) Furukawa, Y., and O'Halloran, T. V. (2006) Posttranslational modifications in Cu,Zn-superoxide dismutase and mutations associated with amyotrophic lateral sclerosis. *Antioxid. Redox Signaling 8*, 847–867.

(12) Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R., and Culotta, V. C. (2001) A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. *J. Biol. Chem. 276*, 38084–38089.

(13) Wood, L. K., and Thiele, D. J. (2009) Transcriptional activation in yeast in response to copper deficiency involves copper-zinc superoxide dismutase. *J. Biol. Chem. 284*, 404–413.

(14) Bush, A. I. (2000) Metals and neuroscience. *Curr. Opin. Chem. Biol. 4*, 184–191.

(15) Cleveland, D. W., and Rothstein, J. D. (2001) From Charcot to Lou Gehrig: Deciphering selective motor neuron death in cells. *Nat. Rev. Neurosci. 2*, 806–819.

(16) Gaggelli, E., Kozlowski, H., Valensin, D., and Valensin, G. (2006) Copper homeostasis and neurodegenerative disorders (Alzheimer's, prion, and Parkinson's diseases and amyotrophic lateral sclerosis). *Chem. Rev. 106*, 1995–2044.

(17) Madsen, E., and Gitlin, J. D. (2007) Copper and iron disorders of the brain. *Annu. Rev. Neurosci. 30*, 317–337.

(18) Lamb, A. L., Wernimont, A. K., Pufahl, R. A., Culotta, V. C., O'Halloran, T. V., and Rosenzweig, A. C. (1999) Crystal structure of the copper chaperone for superoxide dismutase. *Nat. Struct. Biol. 6*, 724–729.

(19) Lamb, A. L., Torres, A. S., O'Halloran, T. V., and Rosenzweig, A. C. (2001) Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. *Nat. Struct. Biol. 8*, 751–755.

(20) Wernimont, A. K., Huffman, D. L., Lamb, A. L., O'Halloran, T. V., and Rosenzweig, A. C. (2000) Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. *Nat. Struct. Biol. 7*, 766–771.

(21) Arnesano, F., Banci, L., and Bertini., I. (2001) Solution structure of the Cu(I) and apo forms of the yeast metallochaperone Atx1. *Biochemistry 40*, 1528–1539.

(22) Lamb, A. L., Wernimont, A. K., Pufahl, R. A., O'Halloran, T. V., and Rosenzweig, A. C. (2000) Crystal structure of the second domain of the human copper chaperone for superoxide dismutase. *Biochemistry 39*, 1589–1595.

(23) Torres, A. S., Petri, V., Rae, T. D., and O'Halloran, T. V. (2001) Copper stabilizes a heterodimer of the yCCS metallochaperone and its target superoxide dismutase. *J. Biol. Chem. 276*, 38410–38416.

(24) Stasser, J. P., Silivai, G. S., Barry, A. N., and Blackburn, N. (2007) A multinuclear copper(I) cluster forms the dimerization interface in copper-loaded human copper chaperone for superoxide dismutase. *Biochemistry 46*, 11845–11856.

(25) Lamb, A. L., Torres, A. S., O'Halloran, T. V., and Rosenzweig, A. C. (2000) Heterodimer formation between superoxide dismutase and its copper chaperone. *Biochemistry 39*, 14720–14727.

(26) Hall, L. T., Sanchez, R. J., Holloway, S. P., Zhu, H., Stine, J. E., Lyons, T. J., Demeler, B., Schirf, V., Hansen, J. C., Nersissian, A. M., Valentine, J. S., and Hart, P. J. (2000) X-ray crystallographic and analytical ultracentrifugation analyses of truncated and full-length yeast

copper chaperones for SOD (LYS7): A dimer-dimer model of LYS7-SOD association and copper delivery. *Biochemistry 39*, 3611−3623.
(27) Caruano-Yzermans, A. L., Bartnikas, T. B., and Gitlin, J. D. (2006) Mechanisms of the copper-dependant turnover of the copper chaperone for superoxide dismutase. *J. Biol. Chem. 281*, 13581−13587.
(28) Kirby, K., Jensen, L. T., Binnington, J., Hilliker, A. J., Ulloa, J., Culotta, V. C., and Phillips, J. P. (2008) Instability of superoxide dismutase 1 of *Drosophila* in mutants deficient for its cognate copper chaperone. *J. Biol. Chem. 283*, 35393−35401.
(29) Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., Zovo, K., and Palumaa, P. (2010) Affinity gradients drive copper to cellular destinations. *Nature 465*, 645−648.
(30) Angeletti, B., Waldron, K. J., Freeman, K. B., Bawagan, H., Hussain, I., Miller, C. C. J., Lau, K. F., Tennant, M. E., Dennison, C., Robinson, N. J., and Dingwall, C. (2005) BACE1 cytoplasmic domain interacts with the copper chaperone for superoxide dismutase-1 and binds copper. *J. Biol. Chem. 280*, 17930−17937.
(31) Zhu, H., Shipp, E., Sanchez, R. J., Liba, A., Stine, J. E., Hart, P. J., Gralla, E. B., Nersissian, A. M., and Valentine, J. S. (2000) Cobalt^{2+} binding to human and tomato copper chaperone for superoxide dismutase: Implications for the metal ion transfer mechanism. *Biochemistry 39*, 5413−5421.
(32) Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) Ellman's reagent: 5,5′-Dithiobis(2-nitrobenzoic acid)—a reexamination. *Anal. Biochem. 94*, 75−81.
(33) Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci. 11*, 2411−2423.
(34) Badarau, A., Firbank, S. J., McCarthy, A. A., Banfield, M. J., and Dennison, C. (2010) Visualizing the metal-binding versatility of copper trafficking sites. *Biochemistry 49*, 7798−7810.
(35) Badarau, A., and Dennison, C. (2011) Copper trafficking mechanism of CXXC-containing domains: Insight from the pH-dependence of their Cu(I) affinities. *J. Am. Chem. Soc. 133*, 2983−2988.
(36) Badarau, A., and Dennison, C. (2011) Thermodynamics of copper and zinc distribution in the cyanobacterium *Synechocystis* PCC 6803. *Proc. Natl. Acad. Sci. U.S.A. 108*, 13007−13012.
(37) Xiao, Z., Donnelly, P. S., Zimmermann, M., and Wedd, A. G. (2008) Transfer of copper between bis(thiosemicarbazone) ligands and intracellular copper-binding proteins. Insights into mechanisms of copper uptake and hypoxia selectivity. *Inorg. Chem. 47*, 4338−4347.
(38) Xiao, Z., and Wedd, A. G. (2010) The challenges of determining metal-protein affinities. *Nat. Prod. Rep. 27*, 768−789.
(39) Xiao, Z., Brose, J., Schimo, S., Ackland, S. M., La Fontaine, S., and Wedd, A. G. (2011) Unification of the copper(I) binding affinites of the metallo-chaperones Atx1, Atox1, and related proteins. *J. Biol. Chem. 286*, 11047−11055.
(40) Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J., and Wedd, A. G. (2004) C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: Sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. *J. Am. Chem. Soc. 126*, 3081−3090.
(41) Hussain, F., Olson, J. S., and Wittung-Stafshede, P. (2008) Conserved residues modulate copper release in human copper chaperone Atox1. *Proc. Natl. Acad. Sci. U.S.A. 105*, 11158−11163.
(42) Stasser, J. P., Eisses, J. F., Barry, A. N., Kaplan, J. H., and Blackburn, N. J. (2005) Cysteine-to-serine mutants of the human copper chaperone for superoxide dismutase reveal a copper cluster at a domain III dimer interface. *Biochemistry 44*, 3143−3152.
(43) Zhou, L., Singleton, C., and Le Brun, N. (2008) High Cu(I) and low proton affinities of the CXXC motif of *Bacillus subtilis* CopZ. *Biochem. J. 413*, 459−465.
(44) Leitch, J. M., Yick, P. J., and Culotta, V. C. (2009) The right to choose: Multiple pathways for activating copper,zinc superoxide dismutase. *J. Biol. Chem. 284*, 24679−24683.
(45) Finney, L. A., and O'Halloran, T. V. (2003) Transition metal speciation in the cell: Insights from the chemistry of metal ion receptors. *Science 300*, 931−936.
(46) Wright, G. S. A., Hasnain, S. S., and Grossmann, J. G. (2011) The structural plasticity of the human copper chaperone for SOD1: Insights from combined size-exclusion chromatography and solution X-ray scattering studies. *J. Biochem. 439*, 39−44.
(47) Ralle, M., Lutsenko, S., and Blackburn, N. J. (2003) X-ray absorption spectroscopy of the copper chaperone HAH1 reveals a linear two-coordinate Cu(I) center capable of adduct formation with exogenous thiols and phosphines. *J. Biol. Chem. 278*, 23163−23170.
(48) Hearnshaw, S., West, C., Singleton, C., Zhou, L., Kihlken, M. A., Strange, R. W., Le Brun, N. E., and Hemmings, A. M. (2009) A tetranuclear Cu(I) cluster in the metallochaperone protein CopZ. *Biochemistry 48*, 9324−9326.
(49) Banci, L., Bertini, I., McGreevy, K. S., and Rosato, A. (2010) Molecular recognition in copper trafficking. *Nat. Prod. Rep. 27*, 695−710.
(50) Laliberté, J., Whitson, L. J., Beaudoin, J., Holloway, S. P., Hart, P. J., and Labbé, S. (2004) The *Schizosaccharomyces pombe* Pccs protein functions in both copper trafficking and metal detoxification pathways. *J. Biol. Chem. 279*, 28744−28755.
(51) Carroll, M. C., Girouard, J. B., Ulloa, J. L., Subramaniam, J. R., Wong, P. C., Valentine, J. S., and Culotta, V. C. (2004) Mechanisms for activating Cu- and Zn-containing superoxide dismutase in the absence of the CCS Cu chaperone. *Proc. Natl. Acad. Sci. U.S.A. 101*, 5964−5969.
(52) Jensen, L. T., and Culotta, V. C. (2005) Activation of CuZn superoxide dismutase from *Caenorhabditis elegans* does not require the copper chaperone CCS. *J. Biol. Chem. 280*, 41373−41379.
(53) Leitch, J. M., Jensen, L. T., Bouldin, S. D., Outten, C. E., Hart, P. J., and Culotta, V. C. (2009) Activation of Cu,Zn-superoxide dismutase in the absence of oxygen and the copper chaperone CCS. *J. Biol. Chem. 284*, 21863−21871.
(54) Barry, A. N., Clark, K. M., Otoikhian, A., van der Donk, W. A., and Blackburn, N. (2008) Selenocysteine positional variants reveal contributions to copper binding from cysteine residues in domains 2 and 3 of human copper chaperone for superoxide dismutase. *Biochemistry 47*, 13074−13083.
(55) Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O'Halloran, T. V., and Culotta, V. C. (1999) Structure-function analyses of the Atx1 metallochaperone. *J. Biol. Chem. 274*, 15041−15045.
(56) Lutsenko, S. (2010) Human copper homeostasis: A network of interconnected pathways. *Curr. Opin. Chem. Biol. 14*, 211−217.
(57) Portnoy, M. E., Schmidt, P. J., Rogers, R. S., and Culotta, V. C. (2001) Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics 265*, 873−882.
(58) White, C., Kambe, T., Fulcher, Y. G., Sachdev, S. W., Bush, A. I., Fritsche, K., Lee, J., Quinn, T. P., and Petris, M. J. (2009) Copper transport into the secretory pathway is regulated by oxygen in macrophages. *J. Cell Sci. 122*, 1315−1321.