The yeast copper chaperone for copper-zinc superoxide dismutase (CCS1) is a multifunctional chaperone promoting all levels of SOD1 maturation

Copper (Cu) is essential for the survival of aerobic organisms through its interaction with molecular oxygen (O₂). However, Cu’s chemical properties also make it toxic, requiring specific cellular mechanisms for Cu uptake and handling, mediated by Cu chaperones. CCS1, the budding yeast (S. cerevisiae) Cu chaperone for Cu–zinc (Zn) superoxide dismutase (SOD1) activates by directly promoting both Cu delivery and disulfide formation in SOD1. The complete mechanistic details of this transaction along with recently proposed molecular chaperone-like functions for CCS1 remain undefined. Here, we present combined structural, spectroscopic, kinetic, and thermodynamic data that suggest a multifunctional chaperoning role(s) for CCS1 during SOD1 activation. We observed that CCS1 preferentially binds a completely immature form of SOD1 and that the SOD1-CCS1 interaction promotes high-affinity Zn(II) binding in SOD1. Conserved aromatic residues within the CCS1 C-terminal domain are integral in these processes. Previously, we have shown that CCS1 delivers Cu(I) to an entry site at the SOD1-CCS1 interface upon binding. We show here that Cu(I) is transferred from CCS1 to the entry site and then to the SOD1 active site by a thermodynamically driven affinity gradient. We also noted that efficient transfer from the entry site to the active site is entirely dependent upon the oxidation of the conserved intrasubunit disulfide bond in SOD1. Our results herein provide a solid foundation for proposing a complete molecular mechanism for CCS1 activity and reclassification as a first-of-its-kind “dual chaperone.”

Copper (Cu)² is required for the activation of dioxygen, a function essential for the survival of aerobic organisms (1).

Oddly enough, the electronic structure of Cu that permits its direct interaction with oxygen also renders it toxic. Cells therefore possess systems to handle the uptake and distribution of Cu to prevent its toxic accumulation, while simultaneously ensuring adequate amounts are available in vivo (2). The discovery of trafficking proteins termed “metallo-chaperones” significantly expanded knowledge of these cellular control systems. Cu chaperones acquire Cu from a membrane transporter, protect it from cytosolic scavenging molecules, and prevent deleterious redox reactions during delivery to various target proteins via specific protein–protein interactions (reviewed in Refs. 3–8).

One prominent Cu chaperone target protein is Cu–Zn superoxide dismutase (SOD1), an abundant homodimeric antioxidant enzyme that detoxifies superoxide anion through redox cycling of its catalytic Cu ion (2O₂⁻ + 2H⁺ → H₂O₂ + O₂ (9)). Newly translated SOD1 is monomeric and inactive (10–12), and conversion to the active homodimer only occurs after several posttranslational modifications (reviewed in Ref. 13). The Cu chaperone for SOD1 (CCS1) mediates at least two of these modifications (14) by engaging nascent SOD1, inserting a Cu ion, and directing disulfide bond formation (15, 16). However, increasing evidence supports additional roles for CCS1 during the SOD1 activation process (17–19). Metallo-chaperones are target-specific, as CCS1 does not recognize other metallo-proteins (reviewed in Ref. 6), whereas Cu chaperones apart from CCS1 cannot activate SOD1 (14, 20).

CCS1 proteins are composed of three domains (D1, D2, and D3), each of which are key for SOD1 activation (20). The N-terminal domain (D1, residues 1–70 in yeast) is required for SOD1 activation under Cu-limiting growth conditions, suggesting that its role may be to acquire Cu from the membrane transporter Ctr1 (2, 20). D1 contains the metal-binding MXCCXC motif and a fold similar to the yeast Atx1 and human Atox1 Cu chaperones that escort Cu from the plasma membrane to the secretory pathway (21–23). The second domain (D2, residues 78–216) possesses sequence and structural homology with SOD1 (21), suggesting that it recognizes SOD1 by mimicking SOD1-SOD1 homodimeric interactions. Supporting this notion,
structures of yeast CCS1 alone (21) and in complex with yeast SOD1 (24, 25) reveal D2-mediated interactions with SOD1. Mutations compromising the CCS1 D2 interface abrogate CCS1-mediated SOD1 activation (26). The C-terminal domain (D3, residues 217–249) contains an invariant CXC motif previously shown essential for complete SOD1 activation (14, 20). Yeast CCS1 proteins completely lacking the D3 CXC motif fail to activate nascent SOD1 or induce oxidation of the SOD1 disulfide bond (20, 27).

Our recently published SOD1-CCS1 heterocomplex structure reveals a previously unobserved β-hairpin conformation of D3 that is stabilized by interactions with residues from SOD1, CCS1 D2, and the linker region between D1 and D2 (25). This novel conformer places the conserved CXC motif near the heterodimeric interface and creates an entry site for Cu delivery during SOD1 activation (25). Further analysis of the SOD1-CCS1 heterodimeric structure is suggestive of a possible 180° rotation of the D3 β-hairpin to transfer Cu(I) from the MXXCXC site in D1 to the SOD1-CCS1 “entry site,” as suggested previously (28). Our subsequent work showed that the D3 β-hairpin enhances the binding affinity to immature SOD1 and widens access for Cu delivery (29).

Contemporary reports from the laboratory of Lucia Banci utilize MS and “in-cell” NMR to promote a mechanism for human CCS1, where D1 alone acquires Cu from the cell and actively delivers Cu to the SOD1 active site (30, 31). Here, CCS1 D3 simply functions as a disulfide isomerase responsible for the oxidation of the SOD1 disulfide bond separately from Cu delivery. Related work from this group points to molecular chaperone-like role(s) for CCS1 upon binding to variant forms of SOD1 (18). The mechanism of SOD1 maturation by CCS1 may not be universal, as further evidenced by the conditional requirement for D1 in yeast (20), but not humans (32), whereas other organisms have CCS1 molecules that lack the MXXCXC motif in D1 (33) or even CCS1 at all (34).

A comprehensive view of CCS1-mediated SOD1 activation is not only valuable for those investigating metallo-chaperones or Cu trafficking but is also potentially beneficial for the SOD1-linked ALS field. ALS is characterized as a protein aggregation–associated fatal neurogenerative disorder, where a fraction of cases are caused by dominant mutations in the gene expressing SOD1 (35, 36). Laboratories characterizing inclusions taken from spinal cords of transgenic mice overexpressing pathogenic SOD1 mutants report that the aggregates are enriched in pathogenic SOD1 proteins lacking Cu and disulfide bonds (37).

The presence of immature SOD1 as the major component of these aggregates suggests that mutant forms of SOD1 may be unable to productively transact with CCS1, leaving them destabilized and aggregation-prone (38). Work by our group and others supports a model where CCS1 possesses both Cu and molecular chaperoning roles that are impeded at specific points in the maturation process by pathogenic ALS mutants (17, 19, 25, 39). However, related studies have purported that molecular chaperone-like functions of CCS1 actually work to target and stabilize disease-causing forms of SOD1 (18, 40). Nevertheless, a detailed biochemical evaluation of these newly discovered molecular chaperoning functions of CCS1 is severely lacking.

**Results**

**Conserved aromatic residues in CCS1 D3 promote binding and activation**

Our recent SOD1-CCS1 heterocomplex structure (25) reveals multiple interactions between CCS1 D3 and the disulfide loop of SOD1 (Fig. S1, A and B). The large indole side chain of Trp-222 stacks with the side chain of Arg-105 from D2 and “anchors” the upstream region of the D3 β-hairpin in a groove between CCS1 D1 and SOD1. Another conserved tryptophan residue (Trp-237) packs into a hydrophobic pocket on D2 and essentially works as a “latch” stabilizing the compact positioning of the D3 CXC motif near the SOD1-CCS1 interface. This significantly expands the SOD1-CCS1 interface beyond that of the SOD1 β-barrel and CCS1 D2 (Fig. S1, C and D). The binding affinities for CCS1 D3 variants W222A and W237A were tested, and removal of either D3 tryptophan similarly hindered SOD1 (E,Zn-SOD1) binding ~4-fold as compared with the WT CCS1 protein measured previously by the same method (Fig. 1A and Table 1) (29). To test the ability of the same CCS1 variants to activate immature SOD1, a 5:1 ratio of Cu(I)-CCS1 to E,Zn-SOD1 WT was mixed at a minimum of 10-fold over the measured $K_D$ in optimized reaction conditions (see “Experimental procedures”). SOD1 activated by Cu(I)-W222A CCS1 showed a decrease in activity to ~30% when compared with...
SOD1 with WT Cu(I)-CCS1, whereas Cu(I)-W237A was completely unable to activate SOD1 (Fig. 1B). Cu occupancy of WT and mutant forms of CCS1 were determined via inductively coupled plasma-MS (ICP-MS) and showed similar binding properties between WT and the two Trp mutants (∼1 Cu monomer). Thus, the data suggest that the stability and correct positioning of the D3 β-hairpin critically effects SOD1 binding and activation by CCS1 with Trp-237 playing a critical role.

The CCS1 D3 CXC motif coordinates Cu(I) before delivery to SOD1

Previous reports from numerous groups have provided conflicting results on the role of the D3 CXC motif in Cu ion coordination for delivery to SOD1 (20, 28, 30). Here, we take advantage of a form of CCS1 that has evolved to contain only the critically conserved D1 MXXCXC and D3 CXC cysteines (tomato CCS1) (41), as opposed to its functional homologues in yeast and humans that have additional cysteines in D1 and D2 (reviewed in Ref. 4). Using the tCCS1 form allows for direct examination of the role for these two motifs in metal binding during SOD1 activation without further perturbation to the protein. Our motivation arises from observations that mutation of the additional cysteines (especially within the D2 β-barrel) of yCCS1 showed dramatic effects on protein folding and stability that would likely affect results in this assay. Here, we show that the WT form of tCCS1 forms disulfide-linked homodimers under nonreducing conditions in vitro (Fig. 2, top row of gels). A D1 MXXCXC (C12A/C15A) mutant behaves similarly to the WT. However, the D3 CXC (C204A/C206A) mutant and a mutant lacking all cysteines did not form dimers even in the presence of copper 1,10-phenanthroline (CuP), which promotes disulfide bond formation. The data show that D3 CXC cysteines can form intersubunit disulfide bonds, likely with the CXC cysteines covalently linking a premade D2-mediated tCCS1 homodimer. When tCCS1 is loaded with Cu(I), the same disulfide linkages do not form. These results strongly advocate that the D3 CXC cysteines participate in Cu(I) coordination and this form of CCS1 can fully activate both immature tomato and yeast SOD1 (Fig. 2, bottom right).



**Table 1**

| Construct                  | $K_D$ (µM) | SOD1 activation |
|----------------------------|-----------|-----------------|
| WT-yCCS1                   | 114.0 ± 22.0 | *****          |
| yCCS1 W222A                | 452.7 ± 55.39 | **             |
| yCCS1 W237A                | 501.4 ± 104.2 | No activation   |
| yCCS1 C16A/C20A            | 228.5 ± 27.74 | *****          |
| yCCS1 C229A/C231A          | 281.0 ± 28.95 | No activation   |
| yCCS1 C16A/C20A/C229A/C231A| 310.1 ± 19.48 | No activation   |
| yCCS1 D3 truncation        | 552.0 ± 105.3 | No activation   |



**Disulfide bond formation drives Cu(I) delivery to the active site**

Based on the obtained Cu(I)-binding affinities, we propose a pathway of Cu delivery to SOD1 that involves the transfer from the 2S site in CCS1 to a Cu ion entry site with trigonal 2S and 1N/O coordination within SOD1-CCS1 (25) and subsequent transfer to the SOD1 active site, where Cu(I) is bound to three Cu ion entry site at the SOD1 active site (X,Zn-SOD1SH) above 1 cm.

**CCS1-mediated Cu(I) delivery is a thermodynamically driven process**

We propose a model in which CCS1-mediated SOD1 activation involves Cu(I) transfer from CCS1 to SOD1 through a Cu ion entry site where it is subsequently shuttled to the SOD1 active site. To verify whether this series of Cu(I) transfer events is thermodynamically driven, we determined the Cu(I)-binding affinity of Cu sites at progressive stages of Cu transfer from CCS1 to SOD1. We used a direct competition assay with two small-molecule Cu(I) chelators (L), BCA and bathocuproine sulfonate (BCS), that allow colorimetric quantification of their Cu-bound species (CuL₂) upon competition with Cu(I)-bound CCS1 or SOD1 (Table 2). The known formation constants for BCA ($\beta_2 = 10^{17.2} M^{-2}$) and BCS ($\beta_2 = 10^{19.8} M^{-2}$) (42) allow for the determination of the Cu(I) dissociation constants for different binding sites on CCS1 and SOD1. To guarantee Cu(I) binding at specific sites in CCS1 and SOD1, site-specific mutants have been utilized. Previous studies, using indirect competition, reported that the Cu(I) affinity of D1 of CCS1 to be an order of magnitude greater than that of D3 (43). However, using a standardized direct competition method in mutants where the Cu(I)-binding residues have been mutated in each domain, we show that the CCS1 D1 and D3 domains possess nearly equal affinity for Cu(I), with $D_1 = 3.61 \pm 1.00 \times 10^{-17} M$ and $D_3 = 2.33 \pm 0.22 \times 10^{-17} M$. On the other hand, the Cu(I) affinity in H46R/H48Q SOD1 (X,Zn-SOD1SH), which contains an ablated active site), where Cu is bound at the entry site (9.00 $\pm 0.14 \times 10^{-19} M$), is nearly 2 orders of magnitude higher than the Cu(I) affinity for either CCS1 site but significantly lower than the Cu(I) affinity at the active site of mature SOD1 (Cu,Zn-SOD1SH), which we determine to be 6.97 $\pm 2.26 \times 10^{-21} M$. Our results indicate that the transfer of Cu between D1 of CCS1 and D3 could involve dynamic Cu(I) equilibrium between their Cu(I)-binding sites. Nevertheless, Cu(I) transfer from CCS1 to the entry site at the SOD1-CCS1 interface and then subsequently to the SOD1 active site is thermodynamically driven by a Cu(I) affinity gradient that allows a series of efficient Cu(I) transfer reactions.
entry site gives rise to a prominent metal-induced shoulder at ~260 nm consistent with CysS-Cu(I) LMCT transition origin. The metal-induced differential absorption for Cu(I) binding to this 2S, IN/O trigonal entry site shows a Δε = 6000 m^−1 cm^−1 consistent with a minor Δε increase compared with Cu(I)-CCS1, likely arising from CysS-Cu(I) LMCT transitions in a changed coordination geometry environment. Removal of non-entry site–coordinating cysteines in SOD1 (Cys-146) and/or CCS1 D1 (Cys-17/Cys-20) does not hinder entry site Cu(I) binding, whereas the mutation of the key entry site cysteines from SOD1 (Cys-57) and/or CCS1 D3 (Cys-231) eliminates all Cu binding away from CCS1 D1 and the SOD1 active site (Table 2 and Fig. S2).

Relatedly, the reaction of Cu(I)-CCS1 with WT E,Zn-SOD1SH leads to significant shift of metal-induced features at 260 nm (with a marked Δε decrease) compared with Cu(I)-Ccs, consistent with the expected shift to higher energy for N/O-Cu(I) LMCT (Fig. 3). This supports the transfer of Cu(I) to the SOD1 active site that is devoid of Cys ligands and for which a 3N/O coordination has been determined by XAS (25) and numerous crystallographic analyses (45, 46).

Considering these differences, stopped-flow spectroscopic kinetic analysis at 260 nm following reactions between Cu(I)-CCS1 and SOD1, under anaerobic and aerobic conditions, monitor the kinetics of Cu transfer from CCS1 to the SOD1 entry site and then to the SOD1 active site. Reaction of Cu(I)-CCS1 with H46R/H48Q SOD1 (X,Zn-SOD1SH) show increasing absorbance traces at 260 nm and are consistent with Cu(I) transfer to SOD1 stalled at the entry site. Results indicate a fast transfer reaction with an apparent second-order k_{app} of ~10^3 M^−1 s^−1 (Fig. 4, bottom). Stopped-flow traces obtained upon mixing Cu(I)-CCS1 and WT E,Zn-SOD1SH in aerobic conditions yielded a fast 260-nm absorbance decrease, suggesting very rapid Cu(I) transfer from CCS1 to the SOD1 active site where Cu(I) is coordinated by histidine residues, with second-order k_{app} of ~10^4 M^−1 s^−1 (Fig. 4, top left). However, similar reactions between Cu(I)-CCS1 and WT E,Zn-SOD1SH in anaerobic conditions showed kinetic traces at 260 nm consistent with the reaction using H46R/H48Q SOD1 (X,Zn-SOD1SH). This is indicative of Cu(I) being stalled at the SOD1 entry site (Fig. 4, top right). CCS1-dependent SOD1 activation is known to require oxygen (15), as now explained by the oxygen-dependent transfer of Cu from the entry site to the SOD1 active site. Further spectroscopic studies performed by mixing Cu(I)-CCS1 and CI46S SOD1 (E,Zn-SOD1SH) mutant incapable of forming the disulfide bond showed a similar electronic absorption envelope as observed for H46R/H48Q SOD1 (X,Zn-SOD1SH), corroborating the Cu stalling at the entry site when the disulfide bond cannot be formed.

**CCS1 interaction stabilizes site-specific high-affinity Zn binding by SOD1**

The acquisition of Zn by SOD1 is not well-understood (reviewed in Ref. 4) and has not been extensively studied (19). The yeast form of CCS1 does not bind Zn on its own (13), and immature SOD1 binds Zn with low affinity where the metal can not be observed.
leach from the protein in the presence of the weak Zn(II) chela-
tor PAR (19, 29). Here, we developed a competition method to
monitor Zn binding in SOD1 using the high-affinity Zn chela-
tor \( \text{N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine} \) (TPEN). After incubation and washing of Zn-bound forms of
SOD1 with competing concentrations of TPEN and subsequent
removal of TPEN–Zn complex, we measured Zn loss in SOD1
by ICP-MS (Fig. 5). The Zn affinity of TPEN is \( 2.6 \times 10^{-16} \text{ M} \) (47). The mature WT form of SOD1 (Cu,Zn-SOD1\(^{SS}\)) holds
onto Zn the most stably (19.28 ± 0.24% Zn loss). The reduced
form of the active site mutant H46R/H48Q SOD1 (X,Zn-
SOD1\(^{SH}\)) showed a significantly reduced Zn(II) binding pro-
pensity (X,Zn-SOD1\(^{SH}\) = 86.47 ± 1.32% Zn loss)). However,
complex formation between the X,Zn-SOD1\(^{SH}\) and CCS1 sig-
ificantly increased the SOD1 affinity for Zn (resulting in
51.94 ± 0.56% Zn loss). Interestingly, X,Zn-SOD1\(^{SH}\) binding by
the CCS1 D3 mutants W222A and W237A, which destabilize the D3 \( \beta \)-hairpin, does not provide the same level of Zn protec-
tion as WT CCS1. In fact, the levels of Zn loss mimic X,Zn-
SOD1\(^{SH}\) alone (86.03 ± 3.27% for W222A and 88.33 ± 2.52% for W237A). CCS1 D3 CXC mutants did not have the same
effect and behaved more similarly to the levels of WT CCS1
protection.

Next, using an in-line equilibrium cell and the known Zn
affinity of TPEN, we developed a competition method with
stringent volume control to, for the first time, determine the Zn
affinity of SOD1 at various stages in maturation (Table 2). Fully
mature WT SOD1 (Cu,Zn-SOD1\(^{SS}\)) binds Zn with the highest
affinity (8.46 ± 2.83 × 10^{-18} \text{ M})). The active-site mutant H46R/
H48Q SOD1 (X,Zn-SOD1\(^{SH}\)) has a significantly weaker affinity
for Zn (1.60 ± 0.24 × 10^{-16} \text{ M}), but upon complex formation
with CCS1, the affinity of X,Zn-SOD1\(^{SH}\) (mimicking immature
SOD1) for Zn increases by nearly an order of magnitude (3.83 ±
0.52 × 10^{-17} \text{ M}). Once again, the Zn affinity values for X,Zn-
SOD1\(^{SH}\) bound by the CCS1 D3 mutants W222A (1.62 ±
0.21 × 10^{-16}) and W237A (1.88 ± 0.79 × 10^{-16}) mimic that
of X,Zn-SOD1\(^{SH}\) without WT CCS1 binding. These \( K_D \) val-
ues substantiate our Zn loss assay results (above) and pro-
mote a mechanism where CCS1 binding to immature SOD1
induces/stabilizes a conformation suitable for stable Zn
binding, thereby accelerating subsequent Cu delivery and
disulfide formation.
Discussion
Our data predict that yeast CCS1 functions beyond a standard metallo-chaperone and promotes all levels of SOD1 post-translation modification. We have shown that CCS1 preferentially binds a completely immature SOD1 molecule (E,E-SOD1<sup>SH</sup>), and this interaction promotes high-affinity Zn binding by SOD1 (Fig. 5 and Table 2). An expanded SOD1-CCS1 interface involving the disulfide loop of SOD1 and the CCS1 D3 β-hairpin probably stabilizes Zn ligand residues in this region (e.g. His-63, His-71, and His-80). Intercalation of the SOD1 disulfide loop by CCS1 D3 concomitantly forms a Cu ion drop-off point or entry site (25). Cu transfer follows a favorable thermodynamic affinity gradient from CCS1 to the SOD1 active site. In conjunction, kinetics show that the transfer process is fast and delivery to the active site is directed by SOD1 disulfide formation. In all, this supports and greatly advances our understanding of the order, rates, and forces guiding the multiple SOD1 maturation events coordinated by CCS1 (25).

It has been generally assumed that SOD1 diffusively acquires Zn from the available pool within the cell as the first step in its maturation process (reviewed in Ref. 13). This is supported by the lack of Zn-specific metallo-chaperones that have been discovered, to date, and the fact that WT SOD1 purified from yeast lacking CCS1 is devoid of Cu, yet enriched with Zn (>1 Zn/monomer) (25). However, this can be misleading, as those SOD1 molecules examined after purification are improperly metallated (i.e. Zn bound in both the Cu and Zn sites) and represent only a portion of the whole SOD1 population, much of which fell within the insoluble fraction as metal-deplete and disulfide-reduced (14, 25). It has also been shown that pathogenic SOD1 variants that cannot productively interact with CCS1, including

Figure 4. Kinetic analysis of CCS1 mediated copper delivery to sites on SOD1. Stopped-flow kinetic traces at 260 nm and corresponding curve fittings (red line) obtained upon rapid mixing of Cu(I)-Ccs (20 μM) with equal volumes of apo H46R/H48Q (X,Zn-SOD1<sup>SH</sup>) or WT E,Zn-SOD1<sup>SH</sup> (20 μM) under aerobic or anaerobic conditions are shown. The decreased absorbance at 260 nm (top left) corresponds to the copper ion moving from the cysteine coordination of CCS1 and/or SOD1 and entering the histidine coordination of the SOD1 active site. Increased absorbance at 260 nm shows the copper ion transfer from CCS1 cysteines to the SOD1-CCS1 2Cys/1His entry site.
D124V and C57S, show improper metallation (i.e., Cu-free and Zn-bound at the Cu site) and are disulfide-reduced (39). Even more recent studies have shown that the major form of SOD1 found within cultured HEK293 cells lacking CCS1 is completely metal-free and disulfide-reduced (30). Taken together, these data argue that CCS1 facilitates proper Zn metallation, although the mechanism of action remains unclear. In support of this model, we have very recently shown that CCS1 recognizes a completely immature form of SOD1 (E,E-SOD1SH), and binding then promotes stable Zn uptake by CCS1-bound SOD1 (29). Our present results provide a molecular basis for proper high-affinity Zn binding by SOD1 that is induced by interaction with CCS1 (Table 2). We find that conserved aromatic residues (Trp-222 and Trp-237) that stabilize the β-hairpin conformation of CCS1 D3 and are critical for overall SOD1 activation are also critical for this function. We have previously asserted (29), and our current data reinforce the notion, that CCS1 D3 stabilizes a loop region of SOD1 containing multiple Zn-liganding residues and generates a complete Zn-binding site in SOD1. It is important to realize that stable Zn binding by SOD1 must occur before Cu insertion and disulfide oxidation can ensue (30, 48). Without CCS1 present, the population of SOD1 disintegrates into an assorted collection of improperly metallated molecules ranging from completely Zn-free to conformers with 2 Zn ions/monomer (reviewed in Ref. 13). CCS1 likely plays an all-encompassing facilitatory role during SOD1 activation that starts with guaranteeing “correct” Zn status of the SOD1 molecule.

The compact β-hairpin conformation of CCS1 D3 expands the SOD1-CCS1 dimeric interface outside that of the SOD1 β-barrel and CCS1 D2 (Fig. S1). Two highly conserved tryptophan residues secure the β-hairpin against CCS1 D2 and place the CXC cysteines near dimeric interface and disulfide cysteines of SOD1, forming what we have termed the Cu ion “entry site.” Trp-222 anchors the first six amino acids of D3 within a narrow groove between SOD1 and CCS1 D1. Within the D3 β-hairpin, Trp-237 interacts with a hydrophobic patch on D2 stabilizing this section of D3 downstream of Trp-222 that is commonly unstructured in the absence of Cu coordination or SOD1 binding (21, 28). Substitution of either residue resulted in a similar ~4-fold loss in binding affinity compared with WT CCS1 (Fig. 1A) (29). These values also compare quite well with a CCS1 truncation that lacks D3 (Table 1) (29), suggesting that without Trp-222 or Trp-237, CCS1 D3 does not complete the observed notch-into-groove interactions guided by the D3 β-hairpin and unfurled SOD1 disulfide loop, as pictured in Fig. S1 and Fetherolf et al. (25).

Removal of these key tryptophan residues in CCS1 D3 significantly hindered SOD1 activation. Both mutants can be loaded in vitro to normal (e.g., similar to WT) levels of Cu binding. The W222A mutant retains some capacity to activate SOD1, but only about one-third that of WT CCS1. The W237A mutant abolishes any SOD1 activation. The lack of cohesion between the Trp-222 and Trp-237 variants where both diter binding ~4-fold, yet their SOD1 activation propensities show some disparity, could be explained kinetically. Whereas both tryptophan residues likely stabilize the positioning of the D3 β-hairpin, without Trp-222, the Trp-237 could likely still find its spot for a subset of CCS1 molecules making the CXC motif available for Cu ion entry site formation. However, without Trp-237, this localization may take much longer or exist less stably. Other related CCS1 variants were tested and are shown in Table 1.

The structural and biochemical framework first generated around our SOD1-CCS1 complex demonstrates a tuned mechanism for the transfer of Cu from CCS1 to the SOD1-CCS1 entry site and subsequent release to the nearby active site. The transient Cu(I) coordination at the entry site is prompted by a ligand-exchange reaction where the introduction of an additional coordinating ligand switches from a 2S digonal Cu(I) site (in CCS1) to a 2S1N/O trigonal environment (in SOD1-CCS1) that results in a nearly 2-order-of-magnitude increase in Cu(I) affinity (Table 2). This resembles processes observed in other Cu-trafficking proteins, where sulfur-coordinated Cu(I) sites with low coordination numbers (2, 3) by “soft” ligands such as Cys (or Met) side chains are selected to guarantee fast ligand exchange between residues of donor and acceptor sites (50).

From the entry site in SOD1-CCS1, Cu(I) is then delivered to the SOD1 active site, where a 3N coordination occurs (25, 30). Based on Pearson HSAB theory (hard and soft, acid and bases), which predicts the soft nature of both Cu(I) and thiolate ligands, it is surprising that the SOD1 active site shows an 80-fold increase in Cu(I)-binding affinity compared with the entry site (with histidine being in nature “harder” than thiols) (51, 52). However, despite the known identity of the Cu(I)-coordinating active-site histidines (His-46, His-48, His-6, and His-120), the exact role of these residues in Cu translocation to the active site remain ambiguous (reviewed in Ref. 13). His-63 also coordinates Zn(II) and can act as bridging ligand, its “nonstandard” side-chain acidity and altered “hardness” could facilitate Cu(I) transfer. His-120 is both an active site and entry site Cu ligand and this may diminish the affinity of the compet-
ing “incomplete” active site (e.g. only 3 free histidines) when liganding Cu(I) as a part of the entry site. Delivery is achieved exclusively when disulfide formation occurs; thus, it appears that stability of Cu(I) bound at the active site in mature SOD1 is modulated by both oxidation of the coordinating thiolate ligand in the entry site (Cys-57) (preventing Cu(I) coordination) as well as possible structural constrains imposed to the active site by the disulfide bridge formation. This fits well with previous work showing that C57S and C146S SOD1 variants isolated from yeast show negligible active site Cu and an overabundance of Zn (~2 Zn ions/SOD1 monomer) (53).

It appears that yeast CCS1 is a multipurpose “helper” protein with conditionally/temporally dependent chaperoning roles during SOD1 activation (Fig. 6). The overall functionality of CCS1 is significantly more complex than that of a standard metallo-chaperone. Related work on the human form of CCS1 has shown an ability to stabilize select disease-causing SOD1 mutants, although any role in SOD1-dependent ALS is unclear (18). Our results support a model where initial binding of immature SOD1 by CCS1 facilitates site-appropriate Zn binding by stabilizing liganding residues present in the disulfide loop of SOD1 (Fig. 6, panels B and C and panels D and E). The second step in the SOD1 maturation process will be dependent on the level of available Cu. During limiting Cu conditions, CCS1 D1 will have likely acquired the necessary Cu(I) from the Cu transporter Ctr1 (54) for delivery to the SOD1 entry site (25) (Fig. 6A). Our previous work has established that at this point, the entry site bound Cu ion will react with a superoxide/peroxide attracted to a nearby electropositive hole and promote sulffenylation at Cys-146 on SOD1 (Fig. 6C) (25). Sulffenylation of Cys-146 prompts disulfide-exchange reactions between nearby cysteines, concluding in formation of the SOD1 intrasubunit disulfide, Cu release into the SOD1 active site (Fig. 6G) (25). Disulfide bond formation also terminates interaction with CCS1, and the mature SOD1 molecule (Cu,Zn-SOD1SS) can find another Cu,Zn-SOD1SS molecule to form the complete homodimeric enzyme (Fig. 6G). When Cu levels are higher, Cu(I)-GSH will likely provide the Cu to CCS1 and/or directly to the SOD1-CCS1 entry site (Fig. 6E). In this instance, CCS1 may serve to stabilize a conformation of SOD1 that displays an available entry site and then that of a disulfide oxidase after Cu drop-off by Cu(I)-GSH (Fig. 6, E–G) (25). In all, CCS1 has a complex mode of action that is honed to specifically activate immature SOD1 during differing cellular conditions in an extremely efficient manner.

**Experimental procedures**

**Materials**

DTT, isopropyl 1-thio-β-D-galactopyranoside, and tris(2-carboxyethyl)phosphine (TCEP) were purchased from GoldBio. Yeast extract, tryptone, NaCl, BisTris, Tris-base, glycine, β-mercaptoethanol (βME), agar, ammonium persulfate, sodium acetate, acetic acid, EDTA, and TEMED were purchased from Sigma-Aldrich. MES, GSH, and trichloroethanol were purchased from Acros Organics. Cu1-(CH3CN4)PF6 was obtained from Strem Chemicals, and 1,10-phenanthroline was from Toronto Research Chemicals. Primers for mutagenesis were purchased from Sigma-Aldrich, and the Phusion site-directed mutagenesis kit was from Thermo Fisher Scientific. TPEN was purchased from Tocris Bioscience. Alexa-546 fluorescent dye for labeling was purchased from Life Technologies. Bacterial strains used were DH5α (Invitrogen), BL21 pLysS (DE3) E. coli (Promega), and XL1-Blue (Stratagene). Chromatography columns were purchased from GE Healthcare. Antibodies used in this study were mouse monoclonal His6 epitope tag antibody (MA1–21315, Invitrogen) and rabbit polyclonal SOD1 antibody (PA1-30195, Thermo Fisher Scientific).

**hSOD1 cloning, expression, and purification**

DNA fragments encoding WT and mutant forms of SOD1 were amplified by PCR and ligated into the YEP351-hSOD plasmid, where expression of the SOD1 protein is directed under
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the control of its own promoter. The protein was expressed, purified, and characterized as described previously (19) with the addition of a DEAE-Sepharose chromatography step between the hydrophobic interaction chromatography and gel filtration column steps. Metal content of purified SOD1 proteins was determined using ICP-MS.

**CCS1 cloning, expression, and purification**

DNA fragments encoding yCCS1 were generated by PCR from plasmids originally supplied by J. S. Valentine (UCLA). CCS1 constructs were cloned into a pkA6H vector, which contains an inducible LacZ promoter, an N-terminal His6 tag, and a tobacco etch virus (TEV) cleavage site. Residues 238–240 of CCS1 D3 were changed to alanine via QuikChange mutagenesis. These substitutions convert the highly conserved 237-WEER240 motif found in CCS1 DIII to WAAA. Alanine at these sites enhanced the yield of CCS1 ~5-fold and also appeared to inhibit the C-terminal proteolysis observed in identically prepared, unsubstituted CCS1 during storage 4 °C. yCCS1 proteins were expressed in *Escherichia coli* BL21 (DE3). Cells containing these expression plasmids were grown in lysogeny broth medium at 37 °C to an A600 of 0.6–0.8. After induction with isopropyl 1-thio-β-D-galactopyranoside (1 mM), the cells were transferred to 30 °C for an additional 4 h before being harvested. yCCS1 proteins were purified using a HisTrap HP Ni2⁺ affinity column purchased from Amersham Biosciences using buffer A (20 mM Tris, pH 8, 300 mM NaCl, and 2 mM DTT) and buffer B (20 mM Tris, pH 8, 300 mM NaCl, 2 mM TCEP, and 1 mM imidazole). The column was washed with 2% buffer B for 10 bed volumes. yCCS1 was eluted with a gradient from 2 to 100% in 80 ml. After purification, the His₆ tag was removed from the CCS1 proteins using TEV protease (A280 = 1 OD) produced in-house and engineered to contain its own non-cleavable His₆ tag. After digestion overnight at room temperature, the cleaved His tag and TEV protease were removed from the CCS1 sample by a final pass through the nickel column. This procedure leaves a two-residue (Gly-His) extension on the CCS1 N terminus. The metal content of purified CCS1 proteins was determined using ICP-MS (Agilent 7900) facility here at UTD. Samples for ICP-MS were digested with 1% HNO₃ for analysis.

**Microplate-based binding assays**

The preparation and completion of the binding assays were as detailed in previous work (49). Here, binding experiments were done with a reaction buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM TCEP, and 0.01% both octyl glucoside and CHAPS. The plates (or gels) were imaged using a GE Typhoon FLA 9500 scanner using filters specific for the fluorophore’s excitation. The binding experiments were completed in replicative quadruplicate on the same plate for comparative and statistical analysis. The fluorescence change was then quantified using ImageQuant TL and then analyzed, and figures were constructed using GraphPad Prism.

**SOD1 activity assays**

Metals were removed from SOD1 by dialysis in 10 mM EDTA, pH 3.8, followed by 1 mM EDTA, pH 5.5. Dialysis in 10 mM EDTA buffer was done for 8 h followed by dialysis in the 1 mM EDTA buffer overnight (all at 4 °C). 0.5 mM EDTA was used to remove trace metals from tubes, tips, and glassware for activation experiments. Cu loading was always performed under anaerobic conditions using a glove box. CCS1 protein was mixed in a 1:1 stoichiometric ratio with Cu(I)-(CH3CN4)PF6 (Strem Chemicals) for 2–3 h in a buffer containing 50 mM Tris, pH 8, 10 mM TCEP, and 150 mM NaCl. Unbound Cu was removed by washing the protein with degassed buffer using a spin filter (EMD Millipore). Activity assays were set up in an aerobic environment with a 5:1 molar ratio of CCS1 to SOD1. The reaction buffer contains 50 mM Tris, pH 8, 100 mM NaCl, 0.5 mM TCEP, 20 μM ZnSO₄, and 200 μM BCS. Samples were incubated for 20 min prior to running the reactions out on an 8% native polyacrylamide gel, followed by visualization using the nitro blue tetrazolium gel method. All experiments were completed in at least three separate gels for comparative analysis.

**Disulfide cross-linking assays**

Tomato Ccs (10 μm) was incubated for 15 min with CuP (25 μm) in reaction buffer (20 mM Tris, pH 7.5, and 300 mM NaCl). Samples were prepared with and without the addition of BME and boiled 10 min prior to visualization on 14% BisTris gels and5mM BCS for WT SOD1 and incubated for 15 h. For Cu(I)-SOD samples, 40 μM samples were reacted with 1 mM BCS for H46R/H48Q SOD1 and5mM BCS for H46R/H48Q SOD1 and150 μM Cu(I)-CCS1 (20 μM) was incubated for 15 min with CuP (25 μM) in reaction buffer (20 mM Tris, pH 7.5, and 300 mM NaCl). Samples were prepared with and without the addition of BME and boiled 10 min prior to visualization on 14% BisTris gels and5mM BCS for WT SOD1 and incubated for 15 h. For Cu(I)-SOD samples, 40 μM samples were reacted with 1 mM BCS for H46R/H48Q SOD1 and incubated for 15 h. The absorbance of the (Cu(I)L₂)₃²⁺ complex formed was then measured using Cary 300 UV-visible spectrophotometer (Agilent) at 562 nm for L = BCA (ε = 7900 M⁻¹ cm⁻¹) and at 483 nm for L = BCS (ε = 13,000 M⁻¹ cm⁻¹). All samples were run in triplicate to ensure proper statistical analysis. The dissociation constants were calculated according to the following, using the formation constants β₂ = 10^{17.2} M⁻² for BCA and β₂ = 10^{19.8} M⁻² for BCA (42).

\[
P - Cu + 2L \rightleftharpoons P + CuL₂
\]

Reaction 1

\[
K_{Dβ₂} = \frac{\left[ P \right]_{total} - 1}{\left[ P - Cu \right] \left[ CuL₂ \right] - 2}
\]

(Eq. 1)

**Detection of Cu transfer from Cu(I)-CCS1 to SOD1**

Samples of SOD1 (20 μM in 20 mM Na₂PO₄, pH 7.6, 50 mM NaCl, 0.5 mM TCEP) were incubated with Cu(I)-CCS1 (20 μM in 20 mM Na₂PO₄, pH 7.6, 50 mM NaCl, 0.5 mM TCEP) for 20
Zn loss assays of SOD1 using TPEN

SOD1 samples (10 μM in 50 mM Tris, 150 mM NaCl) were incubated with 100 μM TPEN (Tocris Bioscience) for 20 min at 25 °C. 3K ultrafiltration spin columns (Sartorius) were used to separate Zn–TPEN complex from SOD1. The SOD1 samples were washed with 12.5 times the reaction volume. Zn–TPEN and Zn bound to SOD1 were determined by measuring Zn concentrations via ICP-MS (Agilent 7900) in the flow-through and supernatant, respectively. Samples for ICP-MS were digested and mixed, and the absorbance was monitored at 260 nm for 300 s at 25 °C. All buffers for this experiment were rendered metal-free by treatment with Chelex resin (Bio-Rad).

Determination of Zn affinity of SOD1, apo-SOD1, and apo-SOD1 in complex with CCS1 by equilibrium dialysis

Zn-binding affinity experiments were performed at pH 7.4 using a Bel-Art in-line equilibrium cell (1 ml). The dialysis membrane was treated with EDTA and boiled to remove any metal and sulfide contaminants. One side of the chamber for each reaction was filled with a solution of TPEN at 100 μM. The other side contained 10 μM protein–Zn complex and 100 μM TPEN. The Zn-exchange reaction was allowed to proceed at room temperature overnight under agitation. ICP-MS was used to analyze the Zn concentrations on both sides. The $K_D$ has previously been determined for TPEN at pH 7.4 to be $2.6 \times 10^{-16}$ (41). We used this value to determine Zn affinity values. All samples were analyzed in triplicate and from multiple preparations to provide meaningful statistical analysis.

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