

Human superoxide dismutase 1 (hSOD1) maturation through interaction with human copper chaperone for SOD1 (hCCS)

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Copper chaperone for superoxide dismutase 1 (SOD1), CCS, is the physiological partner for the complex mechanism of SOD1 maturation. We report an in vitro model for human CCS-dependent SOD1 maturation based on the study of the interactions of human SOD1 (hSOD1) with full-length WT human CCS (hCCS), as well as with hCCS mutants and various truncated constructs comprising one or two of the protein's three domains. The synergy between electrospray ionization mass spectrometry (ESI-MS) and NMR is fully exploited. This is an in vitro study of this process at the molecular level. Domain 1 of hCCS is necessary to load hSOD1 with Cu(I), requiring the heterodimeric complex formation with hSOD1 fostered by the interaction with domain 2. Domain 3 is responsible for the catalytic formation of the hSOD1 Cys-57–Cys-146 disulfide bond, which involves both hCCS Cys-244 and Cys-246 via disulfide transfer.

copper transport | protein maturation | protein-protein interactions

Superoxide dismutase 1 (SOD1) is a well-characterized cuproenzyme that catalyzes the dismutation of the toxic superoxide anion, a byproduct of cellular respiration, to molecular oxygen and hydrogen peroxide [$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$] (1). In mammals, SOD1 is ubiquitously expressed in all tissues and primarily localized in the cytosol, although small amounts are found also in the nucleus, peroxisomes, and intermembrane space (IMS) of mitochondria (2–6). Although mature eukaryotic SOD1 is a remarkably stable homodimer, the nascent monomer requires several posttranslational modifications, including incorporation of zinc and copper ions and the formation of a disulfide bond between Cys 57 and Cys 146, before reaching the active dimeric form (7–10). Despite having a wealth of structural information regarding apo and various metallated forms of SOD1 (11–13), the mechanisms of posttranslational modification are still obscure. Understanding these mechanisms is of critical importance because immature forms of SOD1 are believed to be linked to the neurodegenerative disease familial amyotrophic lateral sclerosis (14–17). Although chaperone-independent maturation of SOD1 has been reported in humans (hSOD1) (18, 19) and other organisms (20–23), the majority of the SOD1 activation within the cell, or 100% of activation in the case of yeast SOD1 (ySOD1), is via the SOD1-specific chaperone protein, copper chaperone for SOD1 (CCS) (24–28). CCS also likely exhibits disulfide isomerase activity (29, 30). However, the molecular mechanism of CCS-dependent maturation of SOD1 is still unknown.

CCS is almost ubiquitously expressed in eukaryotes, with comparable cellular distribution but lower expression levels than SOD1 (27, 31). CCS is a dimeric protein, with each monomer constituted by three distinct domains. The human CCS N-terminal domain 1 (D1) is homologous and shows the same fold of the Atx1-like metallochaperones and harbors the MXCXXC copper-binding motif typical of this family of proteins. Human D1 is essential for hSOD1 copper acquisition in vivo (32), whereas yeast D1 appears to be necessary only under copper-limiting conditions (33). CCS domain 2 (D2) is structurally similar to SOD1 and has been postulated

to be critical for CCS-SOD1 protein recognition (33–35). D2 is unable to bind copper (33); human D2 binds one zinc ion with the same ligands as in SOD1, which are absent in yeast D2. Zinc binding is essential to human CCS (hCCS) function, most likely contributing to protein stabilization (36). CCS domain 3 (D3) is a short polypeptide segment (30–40 amino acids) lacking secondary structure but containing a CXC motif (Cys-244 and Cys-246 in hCCS) essential for CCS-dependent activation of SOD1 (29). The crystal structure of a heterodimeric complex, where an intermolecular disulfide bond links Cys-57 of an inactive mutant of ySOD1 and Cys-229 (Cys-244 in hCCS) of yCCS D3, has been solved (37). It has been proposed that the CCS-dependent SOD1 maturation process involves the interaction of CCS with the reduced disulfide, zinc-bound form of SOD1 protein, leading to the formation of the dimeric Cu,Zn-SOD1 protein, in a process requiring oxygen (29, 38).

Many questions remain unanswered regarding the CCS-mediated SOD1 maturation, (e.g., it is still unknown at what point in the process the proposed intermolecular disulfide bond is formed, if copper and disulfide transfer occurs separately or in a single synergistic step, and what the specific roles of the various domains are). The goal of this work is to elucidate, at the molecular level, the CCS-dependent mechanism of SOD1 maturation. We have addressed this through NMR and electrospray ionization mass spectrometry (ESI-MS) characterization of apo and copper-loaded full-length WT and mutant forms of hCCS, as well as constructs of individual domains of hCCS and their interactions and effect on various metallated forms of hSOD1, with its disulfide bond in either an oxidized or reduced state. We show that hSOD1 reaches its mature copper-loaded form in vitro only when the disulfide-reduced zinc-bound form of hSOD1 is treated with Cu(I)-bound full-length hCCS, whereas truncated forms of hCCS are not able to produce a mature dimeric Cu,Zn-hSOD1 protein. The knowledge acquired using a combination of these complementary techniques (NMR and ESI-MS) allowed us to reinterpret in a comprehensive manner the complete pathway of SOD1 maturation by CCS.

Results

In vitro, hSOD1 reaches its mature, copper-loaded form when the reduced, zinc-containing form of hSOD1 ($\text{E}^*,\text{Zn-hSOD1}^{\text{SH}\ddagger}$) is

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*E indicates the copper-binding site is empty.

[†]A superscripted SH indicates Cys-57 and Cys-146 are both reduced.

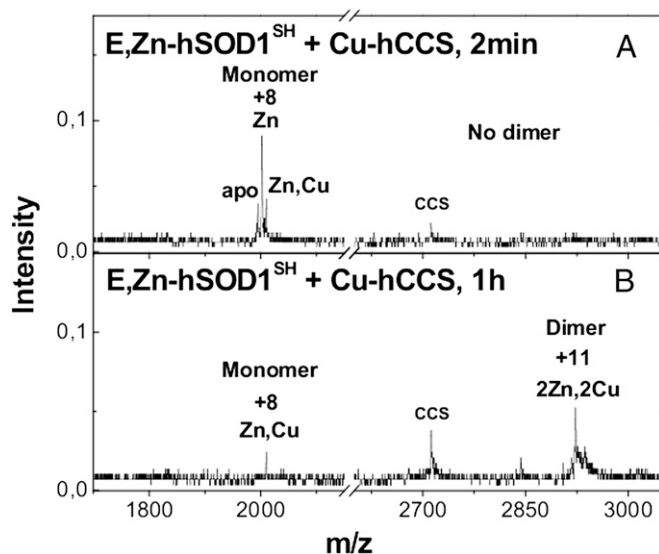


Fig. 1. Incubation of E,Zn-hSOD1^{SH} with full-length Cu(I)-hCCS resulted in mature dimeric Cu,Zn-hSOD1^{S-S}. ESI-MS spectra of E,Zn-hSOD1^{SH} (10 μ M) incubated for 2 min (A) and for 1 h (B) with full-length Cu(I)-hCCS (10 μ M) are shown. Conditions are as follows: 20 mM ammonium acetate, 0.5 mM DTT, pH 7.5, at 25 $^{\circ}$ C. The +8 charge state is presented for E,Zn-hSOD1^{SH} monomer, and the +11 charge state is presented for dimer. The metal content of the protein is also indicated.

treated with full-length Cu(I)-hCCS, resulting in both copper transfer and SOD1 disulfide bond formation. Indeed, incubation of E,Zn-hSOD1^{SH}, which is monomeric, with Cu(I)-hCCS results in the formation of the mature, oxidized dimeric Cu,Zn-hSOD1^{S-S} within 1 h (Fig. 1). Copper transfer was less efficient using the oxidized, zinc-bound form of hSOD1 (E,Zn-hSOD1^{S-S}), which is dimeric, reaching the fully metallated form (Cu,Zn-hSOD1^{S-S}), together with other partially metallated forms, only after incubation for around 13 h (Fig. S1). E,Zn-hSOD1^{SH} also binds Cu(I) when provided as a free ion in solution, leading to Cu,Zn-hSOD1^{SH} after 1 h of incubation. However, no disulfide bond was formed, leaving the protein in the reduced monomeric state (Fig. S2).

Full-length hCCS has previously been reported in a dimeric state (39, 40). Each monomer can bind Cu(I) at two different sites with quite different affinity. Cu(I) binds to the MXCXXC copper-binding motif of D1, a typical Cu(I) chaperone, with a K_d of $2.34 \pm 0.02 \times 10^{-18}$ M, as determined here by ESI-MS using an hCCS construct in which only D1 is expressed (hCCSD1). This value is in agreement with recently published data (40). Cu(I) can also coordinate to the CXC motif of the unstructured D3 (41) but with a much lower affinity with respect to D1 (40). Actually, no binding to an hCCS construct containing D2 and D3 (hCCSD2,3) is observed in the presence of 0.5 mM DTT.

To dissect the mechanism of hSOD1 maturation by hCCS, particularly the role of the three domains during the maturation process, we first analyzed the interactions of various hCCS constructs composed of single (hCCSD1 and hCCSD2) or double (hCCSD1,2 and hCCSD2,3) domains in apo and/or metallated forms, with both oxidized and reduced hSOD1. Neither complex formation nor copper transfer was detected (by ESI-MS) when Cu(I)-hCCSD1 was added to E,Zn-hSOD1^{SH} (Fig. S3), and only partial copper transfer was observed (by NMR) after prolonged incubation (Fig. 2). When E,Zn-hSOD1^{SH} was incubated with Cu(I)-hCCSD1,2, on the contrary, complete copper transfer was observed by NMR (Fig. 2), whereas both copper transfer and formation of a heterodimer complex were

detected by ESI-MS (Fig. S4). Upon titration of E,Zn-hSOD1^{SH} with Cu(I)-hCCSD2,3, a substantial amount of hCCSD2,3 was found in complex with E,Zn-hSOD1^{SH} via ESI-MS but no copper transfer was observed (Fig. 3), indicating that the copper weakly bound to D3 cannot be transferred to SOD1, despite their interaction. Titration of E,Zn-hSOD1^{SH} with hCCSD2 also resulted in the formation of the heterodimeric complex. Therefore, in any CCS/SOD1 mixture in which D2 was present, interaction between monomeric forms of hSOD1 and hCCS, resulting in the formation of 1:1 heterodimeric complexes, was detected by ESI-MS. Disulfide bond formation in hSOD1 and dimerization of protein were not observed for any of these titrations involving single- or double-domain hCCS constructs (Fig. 2C). No copper transfer was observed by NMR when reduced apo hSOD1 (E,E-hSOD1^{SH}), lacking both copper and zinc, was incubated with Cu(I)-hCCSD1,2 (Fig. S5A) even though formation of the heterodimer has been detected via ESI-MS (Fig. S5B).

Overall, these data indicate that all three domains of hCCS are required for obtaining the mature hSOD1: copper transfer from hCCS to hSOD1 is occurring via D1, whereas D2 has a critical role in protein-protein recognition, which is a necessary interaction for D1 to transfer copper to SOD1. Also, zinc binding to hSOD1 before copper binding could be a necessary step to induce a defined and optimal conformation for copper coordination.

The overall behavior is different when various hCCS constructs were titrated with oxidized forms of hSOD1^{S-S}. Copper-loaded hCCS single- or double-domain constructs (hCCSD1, hCCSD1,2, or hCCSD2,3) were either inefficient or totally unable to metallate the oxidized form of E,Zn-hSOD1^{S-S} (Fig. S6). The heterodimeric complex between oxidized E,Zn-hSOD1^{S-S} and hCCSD2,3 was present at a much lower extent compared with incubation with the reduced form (Fig. S6 A and D), indicating that these complexes are highly populated only when the disulfide bond is reduced, as expected. Indeed, E,Zn-hSOD1^{S-S} is predominantly present in the dimeric form; thus, dissociation of the homodimer into monomers able to interact with hCCS may be disfavored. The inability to metallate the oxidized form of hSOD1 reinforces the hypothesis that the posttranslational maturation of hSOD1 is a stepwise process, requiring metallation before disulfide formation. The disulfide bond has a fundamental role in stabilizing the residues in the copper-binding region. The hSOD1 disulfide may prevent copper loading via the CCS-dependent mechanism, because formation of the disulfide bond reduces the accessibility of the metal-binding catalytic site (42). Alternatively, hSOD1 Cys-57 may mediate copper transfer; thus, oxidation may inhibit copper loading.

To dissect the role of the various domains of CCS further, E,Zn-hSOD1^{SH} was titrated with two hCCS constructs either stepwise or simultaneously. E,Zn-hSOD1^{SH} was titrated with Cu(I)-hCCSD1,2, thus obtaining Cu,Zn-hSOD1^{SH}, and was subsequently exposed to air. Even in the presence of oxygen, no formation of the disulfide bond was observed until addition of hCCSD2,3 to the reaction mixture, albeit oxidation was only partial. Similar results were also observed in the absence of air when E,Zn-hSOD1^{SH}, titrated with Cu(I)-hCCSD1,2, was subsequently titrated with oxidized hCCSD2,3 (i.e., the CXC motif of D3 was oxidized). These data indicate that D3 is needed in disulfide bond formation. The role of hCCS D3 in disulfide transfer, as well as D1 in copper transfer, was also confirmed by titrations of E,Zn-hSOD1^{SH} with mixtures of Cu(I)-hCCSD1 and oxidized hCCSD2,3 or with mixtures of hCCSD1 and Cu(I)-hCCSD2,3. Copper transfer and formation of the disulfide bond were observed only in the former case [i.e., in the presence of both Cu(I)-hCCSD1 and hCCSD2,3], although the process was not complete.

To confirm our results regarding the role of D1 in Cu(I) transfer and D3 in disulfide formation, NMR was used to monitor E,Zn-hSOD1^{SH} titrated with full-length Cu(I)-hCCS in which the cysteines of the D3 CXC motif were replaced with Ala either in single C244A and C246A mutations or in a double C244/246A mutation, initially monitored anaerobically. For all three mutants,

[†]A superscripted S-S indicates the presence of the C57-C146 disulfide bond.

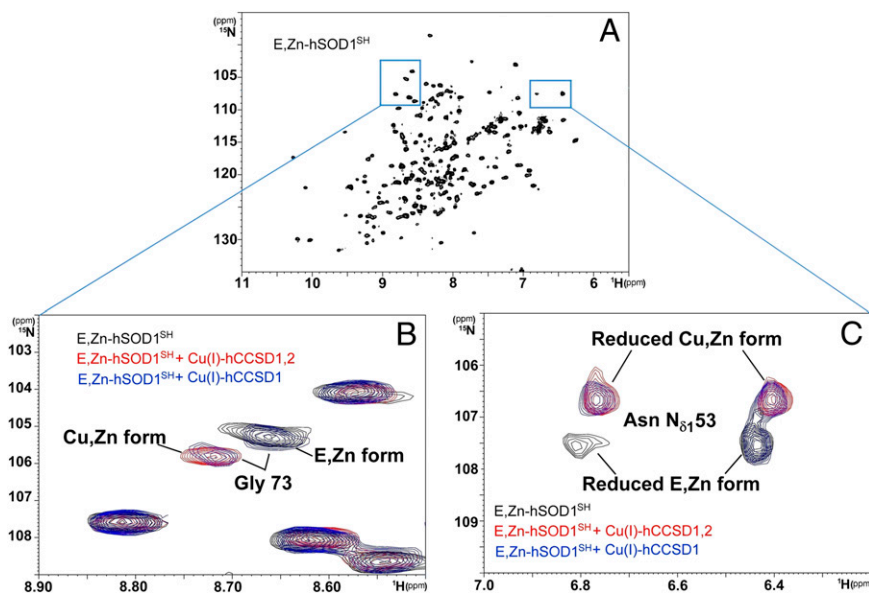


Fig. 2. Transfer of Cu(I) to the hSOD1 is complete in the presence of Cu(I)-hCCSD1,2 and incomplete in the presence of Cu(I)-hCCSD1, with both proteins unable to promote the formation of the disulfide bond of hSOD1. (A) ^1H - ^{15}N heteronuclear single quantum correlation spectroscopy (HSQC) spectrum of E,Zn-hSOD1^{SH}. (B and C) Overlay of selected regions of the ^1H - ^{15}N HSQC spectra of E,Zn-hSOD1^{SH} (black), E,Zn-hSOD1^{SH} in the presence of Cu(I)-hCCSD1,2 (red), and E,Zn-hSOD1^{SH} in the presence of Cu(I)-hCCSD1 (blue). The chemical shift assignments of E,Zn-hSOD1^{SH} and Cu,Zn-hSOD1^{S-S} are available (42, 49). In B, the amide signal of residue Gly-73 is demonstrative of copper binding because it is located in proximity to the catalytic metal-binding site, whereas in C, the chemical shifts of side chain signals of Asn-53 are affected by both copper binding and oxidation. Asn-53 is located in loop 4 of hSOD1 and faces the C57-C146 disulfide on its formation.

Cu(I) transfer was observed but no disulfide formation occurred, with the latter only being partially formed on prolonged incubation (15 h) with air after copper transfer was complete. This is different with respect to what has been observed by NMR in the case of WT full-length Cu(I)-hCCS in a similar experiment, with almost complete oxidation (80%) observed within the 3.75-h time frame of the experiment (Fig. 4). Therefore, it appears that only full-length Cu(I)-hCCS, with the concerted involvement of all three domains, is able to produce mature hSOD1 effectively, starting only from the reduced, zinc-bound form of SOD1.

The midpoint redox potential of the cysteine pairs in hCCSD1 is -243.0 ± 0.5 mV, whereas it is -270.0 ± 1.7 mV for hCCSD2,3 (Fig. S7A). The determination of the midpoint redox potential of E,Zn-hSOD1 and Cu,Zn-hSOD1 was complicated by the occurrence of monomer/dimer equilibrium between the reduced and oxidized species, respectively. From the dependence of intensities of the monomeric peak in ESI-MS spectra, and taking into account the redox potential of the solution, a value of -351.6 ± 1.5 mV was determined for E,Zn-hSOD1 and a value of -337 ± 1.7 mV was determined for Cu,Zn-hSOD1 (Fig. S7 B and C). These reduction potentials are consistent with the role of hCCS as an oxidant of hSOD1.

Discussion

The present work led us to propose an updated model of hCCS-dependent hSOD1 maturation involving at least six steps: zinc acquisition from an unknown source; hCCS-hSOD1 heterodimer formation; hCCS-to-hSOD1 copper transfer via D1; transfer of the hCCS C244-C246 disulfide bond to hSOD1 C57-C146, presumably via formation of an intermolecular disulfide between C244 of hCCS and C57 of hSOD1; and, finally, dimerization of the mature hSOD1 monomer to the active form of the protein (Fig. 5).

Using NMR and ESI-MS methods, and exploiting a number of constructs that feature different combinations of hCCS domains, we have been able to show that only hCCS constructs containing copper-loaded D1 (hCCSD1,2 and, to a lesser extent, hCCSD1) are able to transfer copper to hSOD1, whereas a construct containing copper-loaded D3 (hCCSD2,3) was not. No copper transfer from constructs containing copper-loaded D1 to constructs containing D3 was observed. This observation is fully consistent with the much higher Cu(I) affinity of D1 with respect to that of D3, which thermodynamically disfavors Cu(I) transfers from D1 to D3 (40). The metal transfer is efficient and complete only in the presence of D2, because D1 alone is unable to form

the complex with SOD1 required to facilitate copper transfer. This is a different behavior with respect to copper proteins that receive the metal from Atx1-like chaperones, which show the same fold and Cu(I)-binding site of hCCSD1. However, the partners of the Atx1-like chaperones are completely different from SOD1, not only in terms of overall fold but in terms of the metal coordination sites and their solvent accessibility (43–46). hCCSD2, in addition to possessing the same fold of SOD1, contains a hydrophobic region of the β -barrel that is perfectly suited to interact with the hydrophobic region of the β -barrel of the SOD1 monomer. This interaction would then bring hCCSD1 into an optimal orientation for copper transfer. Finally, oxidation of the hSOD1 intramolecular disulfide bond occurs only in the presence of D3.

Furthermore, using NMR to compare the maturation of hSOD1 utilizing both WT full-length hCCS and Cys-to-Ala mutants of D3, we have shown that when the CXC motif is compromised, Cu(I) transfer is maintained, whereas disulfide formation is negated. This confirms that D1 is responsible for Cu(I) transfer during hCCS-dependent hSOD1 maturation. These results also indicate that both cysteines in the D3 CXC motif are required for disulfide isomerase activity, because both the single C244A and C246A mutants reduce the rate of Cu,Zn-hSOD1^{SH} oxidation similar to the double-C244/246A mutant (Fig. 4B). This claim is further supported by titrations of Cu,Zn-hSOD1^{SH} with oxidized hCCSD2,3, where the disulfide in the CXC motif is transferred to hSOD1 presumably via the formation of an intramolecular disulfide bond between hCCS C244 and hSOD1 C57 as previously reported in the yeast system (29). We determined the reduction potential for E,Zn-hSOD1 and Cu,Zn-hSOD1 to be -351.6 ± 1.5 mV and -337 ± 1.7 mV, respectively. Although the reduction potential of the cellular compartments where hSOD1 is found, cytoplasm and the mitochondrial IMS, should be able to oxidize the disulfide bond (47), we have observed that in the absence of hCCS (Fig. 4), the oxidation process in vitro is kinetically slow, as previously reported for yeast (29). This suggests that SOD1 disulfide formation is under kinetic control. The specific protein-protein interaction between one subunit of hCCS and one subunit of hSOD1 might be crucial not only for copper(I) transfer but for the disulfide exchange reaction. Protein-protein interactions could position Cys57 of the disulfide-reduced hSOD1, located in a solvent-exposed loop at the heterodimer interface, in the correct orientation to promote the formation of an intermolecular disulfide bond with CCS D3, which may then rapidly evolve in the formation of the intramolecular bond.

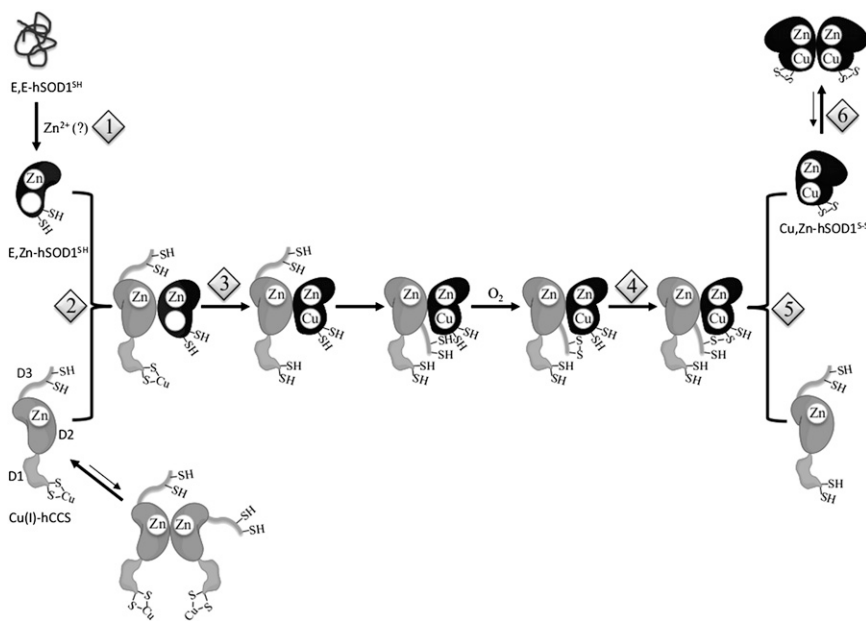


Fig. 5. Schematic mechanism of CCS-dependent hSOD1 maturation in vitro. (1) Nascent hSOD1 (E,E-hSOD1^{SH}) acquires zinc from an unknown source, producing E,Zn-hSOD1^{SH}. (2) The hSOD1^{SH} monomer forms a heterodimer with a monomer Cu(I)-hCCS. The current study suggests that copper transfer (3) occurs before formation of the intermolecular (4), and then hSOD1 intramolecular (5), disulfide bond, resulting in the mature monomer (6) that dimerizes to the active state. Although oxygen in vitro is required in the process before step 4, it is currently unknown precisely at which step this occurs. [Note: E,Zn-hSOD1^{SH} can obtain Cu(I) in a CCS-independent manner.]

for SOD1 maturation, the necessity for and role of D1 remained inconclusive (30, 32, 33, 41, 48). The previous reported findings were principally based on assays measuring for overall SOD1 activity (which requires complete SOD1 maturation); thus, they were unable to monitor separately for copper acquisition and disulfide bond formation, as accomplished in this work using NMR.

Based on our findings, and consistent with what has been reported in the literature, we can propose that the SOD1 dependence on D3 for maturation is the result of its role in disulfide isomerase activity and not a copper transport function, whereas D1 is responsible for Cu(I) insertion. These results are particularly interesting considering that some organisms lack the D1 MXCXXC copper-binding motif, including *Drosophila melanogaster* and *Anopheles gambiae* (23) and the fission yeast *Saccharomyces pombe* (21). Because CCS from *D. melanogaster* is able to activate *D. melanogaster* SOD1 and γ SOD1 fully without exhibiting copper chaperone activity (23), it appears that the primary function of CCS is that of a disulfide isomerase. This then poses the question of how SOD1 is obtaining copper in the absence of a functional D1. We speculate that the most likely answer is via the same mechanism as in the CCS-independent maturation of SOD1, which is currently attributed to a copper–glutathione complex as observed in vitro in yeast (27). Also, human CCS-independent SOD1 maturation is known to require glutathione (19). Thus, there are at least three mechanisms for SOD1 maturation: a first mechanism whereby SOD1 is dependent on CCS for both copper transfer (via D1) and disulfide transfer (via D3), such as is observed for yeast under copper-limiting conditions (33); a second mechanism whereby SOD1 acquires copper from another source and then the disulfide from CCS (via D3), such as is likely for *D. melanogaster* (23); and a third mechanism comprising a completely CCS-independent system, such as is observed for *Caenorhabditis elegans*, which lacks CCS altogether (24), as well as in organisms in which the chaperone-dependent systems are also present (19). For organisms in which both CCS-dependent and independent mechanisms have been described, such as for humans, CCS-dependent maturation is more efficient (22).

This work is a study of the maturation of hSOD1 via hCCS at the molecular level. Using a combination of NMR and ESI-MS, we have elucidated various steps in the process of CCS-dependent maturation of hSOD1, specifically the roles of the individual domains during copper acquisition and disulfide formation processes.

Materials and Methods

hSOD1, Full-Length hCCS, and hCCS Constructs. Expression of full-length hCCS and constructs, as well hSOD1, including metallation techniques, is described in *SI Materials and Methods*.

ESI-MS Measurements for Protein-Protein Interactions and Copper-Binding Affinity. For all protein samples, including hSOD1, full-length CCS, and constructs, protein-protein interactions were monitored after various time points from 2 min to 13 h, depending on the experiment. For copper determination of copper-binding affinity, increasing concentrations of DTT were added to 10- μ M samples of the apo or Cu(I)-bound form of hCCSD1 and hCCSD2,3 constructs. Samples were then incubated for 2 min and finally analyzed by ESI-MS. Further experimental details and instrument settings are available in *SI Materials and Methods*.

Determination of Midpoint Redox Potential of Various CCS Domains, E,Zn-hSOD1, and Cu,Zn-hSOD1. For determination of the midpoint redox potential for hCCSD1 and hCCSD23, the fully reduced hCCS domains were incubated in β -mercaptoethanol-based (BME) redox buffers at room temperature for 1 h, which was sufficient to reach the redox equilibrium between the oxidized and reduced protein forms. The BME buffer with a total concentration of 5 mM BME was used. For determination of the midpoint redox potential for E,Zn-hSOD1 and Cu,Zn-hSOD1, the fully oxidized proteins were incubated in DTT-based redox buffers at room temperature for 1 h with a total concentration of 5 mM DTT. Further experimental details, instrument settings, and mathematical determination of midpoint redox potentials using the Nernst equation are given in *SI Materials and Methods*.

NMR Experiments. The oxidized or reduced form of E,E-hSOD1 and E,Zn-hSOD1 was titrated with unlabeled full-length hCCS or constructs and monitored by ¹H-¹⁵N heteronuclear single quantum correlation spectroscopy NMR. The chemical shift assignments of E,Zn-hSOD1^{SH} and Cu,Zn-hSOD1^{S-S} are available (42, 49). Details regarding instrumentation and data processing are given in *SI Materials and Methods*.

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