A molecular chaperone activity of CCS restores the maturation of SOD1 fALS mutants

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Superoxide dismutase 1 (SOD1) is an important metalloprotein for cellular oxidative stress defence, that is mutated in familiar variants of Amyotrophic Lateral Sclerosis (fALS). Some mutations destabilize the apo protein, leading to the formation of misfolded, toxic species. The Copper Chaperone for SOD1 (CCS) transiently interacts with SOD1 and promotes its correct maturation by transferring copper and catalyzing disulfide bond formation. By in vitro and in-cell NMR, we investigated the role of the SOD-like domain of CCS (CCS-D2). We showed that CCS-D2 forms a stable complex with zinc-bound SOD1 in human cells, that has a twofold stabilizing effect: it both prevents the accumulation of unstructured mutant SOD1 and promotes zinc binding. We further showed that CCS-D2 interacts with apo-SOD1 in vitro, suggesting that in cells CCS stabilizes mutant apo-SOD1 prior to zinc binding. Such molecular chaperone function of CCS-D2 is novel and its implications in SOD-linked fALS deserve further investigation.
Here, we investigated further how CCS exerts this protective role. We characterized by in-cell NMR the interaction between CCS and SOD1, both WT and the WT-fALS mutants A4V, T54R, G93A, I113T, in the cytoplasm of living human cells. We showed that the SOD-like domain of CCS, D2, acts as a molecular chaperone in the human cytoplasm. D2 alone is sufficient to interact with the immature, metastable state of fALS SOD1 mutants, and effectively prevents the accumulation of unstructured species. In the absence of D1 and D3, the CCS-dependent maturation cannot occur and the SOD1-D2 heterodimer becomes a stable intermediate, whereas full-length CCS interacts transiently with SOD1 allowing its complete maturation.

In vitro analysis revealed that, in the heterodimer observed in cells, SOD1 is in the zinc-bound form. These data suggest that D2 of CCS favours zinc binding to mutant SOD1 by interacting either with the zinc-bound protein and preventing zinc dissociation, or with the apo protein stabilizing it and allowing zinc binding. In favour of the latter hypothesis, we further showed that D2 of CCS is able to interact with apo-SOD1 in vitro, and therefore that CCS acts as a molecular chaperone towards the most immature state of SOD1.

Results

CCS-D2 forms a stable heterodimer with zinc-bound WT SOD1. WT SOD1 overexpressed in the human cell cytoplasm in the presence of excess zinc is found in a stable intermediate maturation state, i.e. a homodimer with one zinc ion bound to each monomer, and all cysteines reduced (henceforth E,Zn-SOD1SH)25. This species is free to tumble in the cytoplasm, and gives rise to well resolved signals in the 1H-15N in-cell NMR spectra (Fig. 1a). When full-length CCS (FL-CCS) is co-expressed at similar levels, SOD1 maturation proceeds further along its pathway: the oxidized zinc-bound SOD1 (E,Zn-SOD1SS) is observed in excess of copper, whereas in excess of copper the fully mature protein (Cu,Zn-SOD1SS) is observed25. CCS is not visible in the 1H-15N in-cell NMR spectra, with the exception of a few signals (Supplementary Figure S1a), due to interactions with large cellular components such as the plasma membrane29, but is clearly observed in the NMR spectra of the cell lysates (Supplementary Figure S1b).

When CCS-D2 was co-expressed with WT SOD1 (95.7 ± 2.6 µM and 118.2 ± 5.4 µM, respectively), the resulting in-cell NMR spectra were remarkably different from those of E,Zn-SOD1SH, suggesting the formation of a stable complex between SOD1 and CCS-D2 (Fig. 1a). An overall line broadening was observed, and a few signals of SOD1 were shifted. Several more signals arising from CCS-D2 were also present. The line broadening observed in the in-cell NMR spectra likely arises from the residual interactions of the CCS-D2 monomer with the plasma membrane; consistently, CCS-D2 expressed alone was not detected by in-cell NMR (Supplementary Figure S1c,d) due to such interactions. Upon cell lysis, the complex between SOD1 and CCS-D2 remained unaltered and gave rise to sharp signals in the lysate NMR spectra, with no obvious chemical shift changes with respect to the in-cell NMR spectra (Fig. 1b).

Comparison with in vitro NMR spectra acquired on [U-15N]-E,Zn-SOD1SH titrated with 1 equivalent of unlabelled CCS-D2 (Fig. 1c) confirmed that the intracellular species observed is indeed the complex between E,Zn-SOD1SH and CCS-D2. The crosspeaks arising from SOD1 in the complex formed in vitro were in slow...
bond formation mediated by CCS28. Interestingly, T54R SOD1 also formed a stable complex with CCS-D2 also investigated. The T54R mutation does not cause the misfolding of SOD1, but interferes with the disulfide caused the disappearance of several well-dispersed NMR signals of the monomeric apo-SOD1SH, together with three equivalents of unlabelled CCS-D2 and monitored by NMR after each addition. The addition of CCS-D2 (Supplementary Figure S3d).

Two additional signals were observed in the NMR spectra of the lysate arising from an excess of free CCS-D2 homodimer (Supplementary Figure S3). Also in this case the complex was unaltered upon cell lysis, and no additional species was greatly decreased in the in-cell NMR spectra and the signals arising from the heterodimer with A4V SOD1 (61.8 ± 2.1 µM) compared to co-expressed CCS-D2 (83.1 ± 8.5 µM) and to the other mutants, additional signals were observed in the NMR spectra of the lysate arising from an excess of free CCS-D2 homodimer (Supplementary Figure S3d).

The interaction between CCS-D2 and T54R SOD1 (80.4 ± 6.5 µM and 85.2 ± 10.0 µM, respectively) was also investigated. The T54R mutation does not cause the misfolding of SOD1, but interferes with the disulfide bond formation mediated by CCS28. Interestingly, T54R SOD1 also formed a stable complex with CCS-D2 (Supplementary Figure S4), indicating that the mutation did not affect the interaction with D2 (as it would be expected from the structure reported by Lamb et al. 19), but likely interferes with the D3-mediated disulfide bond formation (consistent with a more recent report by Fetherolf et al. 24).

Overall, these data indicate that CCS-D2 interacts with fALS SOD1 mutants in a similar fashion to the WT SOD1. CCS-D2 forms a stable heterodimer with mutant SOD1 that is structurally equivalent to the heterodimer with the zinc-bound WT SOD1. By doing so, CCS-D2 prevents the formation of the unstructured species in the cells, effectively acting as a molecular chaperone.

CCS-D2 interacts with WT apo-SOD1SH in vitro. In order to further elucidate the mechanism of action of CCS in stabilizing SOD1, we sought to determine whether CCS-D2 could also interact with the disulfide-reduced apo form of SOD1. To this aim, WT [U-15N]-apo-SOD1SH was titrated in vitro with up to three equivalents of unlabelled CCS-D2 and monitored by NMR after each addition. The addition of CCS-D2 caused the disappearance of several well-dispersed NMR signals of the monomeric apo-SOD1SH, together with the appearance of broader peaks presumably arising from apo-SOD1SH in the complex with CCS-D2 (Fig. 3a). With 1 equivalent of CCS-D2, ~20% of free apo-SOD1SH was still detected; the latter disappeared only in the presence of more than 2 equivalents of CCS-D2, suggesting that its affinity for CCS-D2 is lower than that of E,Zn-SOD1SH (Fig. 3b,c).

For clarity, within the text box:
15N relaxation analysis performed on the final 1:3 mixture of apo-SOD1SH and CCS-D2 resulted in an estimated $\tau_m$ of 18.6 ± 1.4 ns, obtained by analyzing a set of well-dispersed signals arising mainly from residues in the β-barrel of SOD1 (shown in Fig. 3a). Such value is comparable to that of the complex between E,Zn-SOD1SH and CCS-D2, and is markedly increased from the $\tau_m$ reported for the monomeric apo-SOD1SH (10.3 ± 0.4 ns)13.

In order to assess whether apo-SOD1SH in the complex with CCS-D2 could bind zinc, the apo-SOD1SH – CCS-D2 complex was further titrated with 1 equivalent of zinc per SOD1 monomer. The NMR spectra of the final point of the titration revealed that the E,Zn-SOD1SH – CCS-D2 complex had formed (Supplementary Figure S5a), identical to that obtained by direct titration of E,Zn-SOD1SH with CCS-D2 (Supplementary Figure S5b and Fig. 1c), meaning that interacting with CCS while in the apo state would eventually lead SOD1 towards the same maturation pathway observed for the zinc-bound form.

Taken together, these data are consistent with the formation of a heterodimer between apo-SOD1SH and CCS-D2 with the same size as that between E,Zn-SOD1SH and CCS-D2. Therefore, CCS-D2 can also bind the non-metallated form of SOD1, albeit with lower affinity, possibly contributing to the stabilization effect observed in cells, and allowing zinc binding to occur subsequently on the apo-SOD1SH – CCS-D2 complex.

**Discussion**

fALS-linked SOD1 mutations are known to have several detrimental effects on the folding stability and on the maturation pathway of SOD1. In particular, several wild type-like mutations have been associated to a thermodynamic destabilization of SOD1, and have been shown to decrease the conformational stability of the apo, disulfide-reduced protein11,30. Furthermore, some mutants have been shown to destabilize the homodimer, due to a reduced association constant for the dimer formation31,32. The latter effect contributes to shift the equilibrium in cell towards the monomeric state, while the former increases the rate of the formation of the unstructured species. The interaction between SOD1 and CCS – mediated by D2 – has been extensively investigated before15–24, and CCS is known to have a stabilizing effect on the maturation pathway of SOD1: overexpression of CCS in cell cultures and transgenic mice has previously revealed a protective function against the aggregation of mutant SOD133–35. Traditionally, the ability of CCS to rescue SOD1 from unfolding and aggregation has been attributed to its metallochaperone and oxidoreductase activities (i.e. copper transfer and formation of the disulfide bond), and D2, the SOD-like domain of CCS, has been relegated to a simple recognition function, required to allow the formation of the transient SOD1-CCS complex. Here we show that CCS-D2 forms a stable complex with mutant SOD1, and by doing so it prevents the accumulation of unstructured apo-SOD1 in the cytoplasm. Our observations are consistent with the protective role of full-length CCS previously observed in vivo31–35. Compared to SOD1, CCS is expressed at lower levels in living organisms36, but its functional complex with immature SOD1 is transient and leads to the formation of fully mature SOD1, allowing CCS to be recycled and interact with a new molecule of immature SOD1. Unlike full-length CCS, the complex between SOD1 and CCS-D2 obviously cannot proceed towards fully mature SOD1 and becomes the end point of the pathway. Such complex is clearly observed by in-cell NMR and corresponds to a SOD1 dimer-like heterodimer containing a SOD1 and a CCS-D2 subunit, relatively free to move in the cytosol. Notably, the SOD1 mutants in the complex with CCS-D2 have bound a zinc ion, and are structurally equivalent to WT E,Zn-SOD1SH in the same complex. Therefore, the stabilizing effect of CCS-D2 on mutant SOD1 is twofold: it prevents the accumulation of unstructured apo protein and facilitates zinc binding.
Based on these observations and on those previously reported, we can summarize the pathway required for SOD1 maturation, and qualitatively indicate in which step CCS will positively affect the pathway (Fig. 4). We can hypothesize two possible modes of actions for the stabilizing effect of CCS-D2, which are not mutually exclusive: one is that zinc binding to SOD1 occurs first, and then CCS interacts with E,Zn-SOD1\(^{15}\) (Fig. 4a). This pathway would be most consistent with what observed for WT SOD1, as it is known to bind zinc spontaneously in the cytoplasm. In the case of mutant SOD1 however, it would imply that the protein would bind zinc and then, in the absence of CCS, lose it.

The other mode of action is that CCS first interacts with monomeric apo-SOD1\(^{15}\) and stabilizes it in a homodimer-like folding state, and then apo-SOD1 in the heterodimer binds zinc (Fig. 4b). In order to determine whether this mechanism occurs in the cell, we would have to separate in time the formation of the heterodimer and the zinc binding. However, this is difficult to achieve because cells have to be supplied with zinc during protein expression, due to the fact that also CCS-D2 needs zinc to fold correctly. In vitro, on the other hand, the two events are easily separated as zinc-containing CCS-D2 can be mixed with apo-SOD1\(^{15}\). Our data show that CCS-D2 indeed interacts with apo-SOD1\(^{15}\) and forms a heterodimer in vitro, albeit with lower affinity than with E,Zn-SOD1\(^{15}\). The NMR signals arising from the folded part of free apo-SOD1\(^{15}\) appeared to be in a slow-intermediate exchange regime with the corresponding signals in the complex, implying a dynamic equilibrium in the milliseconds time scale, slow enough to allow the conformational rearrangement of SOD1 as a consequence of zinc binding. While the interaction between apo-SOD1\(^{15}\) and CCS-D2 had been reported before\(^{22,23}\), its functional role had not been fully recognized and deserves further characterization.

In this work, we showed that the SOD-like domain of CCS acts as a molecular chaperone towards immature SOD1 and helps to stabilize the folded state of fALS-linked wild type-like SOD1 mutants, favouring their correct maturation within the cell. CCS-D2-mediated stabilization has a twofold effect: it prevents the accumulation of unstructured apo species of SOD1, which are the potential precursors of pathogenic forms such as oligomers or aggregates, and allows SOD1 to bind zinc, which is a critical step of its maturation towards the enzymatically active and structurally stable protein. We showed that in vitro CCS-D2 can interact either with apo-SOD1\(^{15}\) or with E,Zn-SOD1\(^{15}\), with higher affinity for the latter, with which it produces a stable complex that is also observed in living cells. While both mechanisms would allow SOD1 to progress through the correct maturation pathway, the direct interaction between CCS and apo-SOD1\(^{15}\) gives credit to the hypothesis that CCS stabilizes SOD1 prior to zinc binding. The molecular chaperone role of CCS described here adds further depth to our understanding of how this protein is critical for the correct maturation of SOD1, and will likely have important implications in the context of developing novel strategies for preventing the cytotoxic effects of mutant SOD1 in familial ALS.

**Methods**

**Gene cloning.** The cDNA encoding the SOD-like domain of human CCS (amino acids 84–234, GenBank: NP_005116.1) was amplified by PCR using the primers D2-F (5′-CGGGATCCCATGCAGAATCTGGGGGCAGTGGCC-3′) and D2-R (5′-ATATCGCCGTCGAGAAGCTTCTATTTAAGCGGAGGCATATGGATGCGCAAG-3′), and cloned into the pHSec\(^{37}\) vector between EcoRI and XhoI restriction enzyme sites to generate the mammalian expression plasmid. The clone was verified by DNA sequencing. pHSec vectors encoding WT human SOD1 (amino acids 1–154, GenBank: NP_000445.1) and the mutants A4V, T54R, G93A and I113T had been generated previously\(^{28}\).
Human cell culture and transfection. HEK293T (ATCC CRL-3216) cells were maintained in DMEM high glucose (Life Technologies) supplemented with L-glutamine (Life Technologies, 100 U/ml final concentration) and 10% FBS (Gibco) in uncoated 75 cm² plastic flasks and incubated at 37°C, 5% CO₂ in a humidified atmosphere. Cells were transiently transfected with the pHsec plasmid containing the gene of interest using polyethylenimine (PEI, branched, average Mₐ 25 kDa, Sigma-Aldrich), as previously described[18]. For co-expression of SOD1 (both WT and mutants) and CCS-D2, cells were transfected with plasmids containing the constructs in different amounts and ratios. The final ratio was chosen in order to obtain similar expression levels of the two proteins; specifically a weight ratio of 1:1:2 (25 µg SOD1 WT or mutant DNA; 25 µg CCS-D2 DNA; 50 µg PEI per 75 cm² flask) was used. [U-[¹⁵N]-BioExpress6000 medium (Cambridge Isotope Laboratories) was used for in-cell NMR samples, supplemented with 2% FBS, antibiotics and 10µM of ZnSO₄.

In-cell NMR sample preparation. Samples for in-cell NMR were prepared following a reported protocol[1]. Briefly, transfected cells were detached with trypsin, suspended in DMEM + 10% FBS, washed once with PBS and re-suspended in one pellet volume of DMEM supplemented with 90 mM glucose, 70 mM HEPES and 20% D₂O. The cell suspension was transferred in a 3 mm Shigemi NMR tube, which was gently spun to sediment the cells at the bottom. Cell viability before and after NMR experiments was assessed by trypan blue staining. After the NMR experiments, the cells were collected and the supernatant was checked for protein leakage by NMR. The cell lysates were prepared by freeze-thaw cycles in PBS buffer followed by centrifugation to remove the insoluble fraction. The supernatant was collected for NMR analysis.

Expression and purification of SOD1. WT SOD1 protein was prepared following an existing protocol[19]. Briefly, a cell culture of E. Coli BL21(DE3) Gold (Stratagene), transformed with a pET28a plasmid containing the WT SOD1 gene was grown overnight at 37°C in LB, harvested, and re-suspended in ¹⁵N-labelled M9 medium. ZnSO₄ was added in the culture to a final concentration of 100 µM. After 4h from induction with 0.5 mM IPTG at 30°C the cells were harvested and re-suspended in 20mM Tris, pH 8 buffer for lysis. The cleared lysate was loaded on an anion exchange column (HiPrep Q FF 16/10, GE Healthcare) for a first purification by elution with NaCl gradient. The fractions containing SOD1 (checked by SDS-PAGE) were collected and further purified by gel filtration (Superdex 75 26/60 column, GE Healthcare) in 20 mM Tris, 100 mM NaCl, pH 8 buffer. Fractions containing pure SOD1 were collected. 1mM DTT was added in all buffers to prevent protein aggregation mediated by disulfide bonds.

Apo-SOD1[19] was produced by repeated dialysis against 10 mM EDTA in 50 mM acetic acid at pH 3.5. Then, the buffer was exchanged in phosphate-buffered saline (PBS) pH 7.4. To obtain apo-SOD1[20], apo-SOD1[21] was incubated 40 min at 37°C with 50 µM of DTT. Following disulfide bond reduction, the buffer was exchanged to remove DTT and dissolved oxygen. E-Zn-SOD1[20] was then obtained by adding one equivalent per monomer of ZnSO₄ in anaerobic conditions. Correct disulfide reduction and metallation were checked by [¹H-¹⁵N] NMR.

Expression and purification of CCS-D2. The synthetic gene encoding CCS-D2 cloned in the pTH34 plasmid with an N-terminal histidine tag was obtained previously[22]. The protein was expressed in E. coli BL21(DE3) C41 cells (Stratagene). Protein expression was induced with 0.7 mM IPTG for 16 h at 30°C. ZnSO₄ was added in the culture to a final concentration of 100 µM. Purification was performed using a nickel-chelating HiTrap (GE Healthcare) column. After digestion with AcTEV protease (Invitrogen) O/N at 25°C the protein was separated from the affinity tag in a HiTrap column. To further separate CCS-D2 from the residual affinity tag, the digested protein was loaded on a Superdex 75 26/60 (GE Healthcare) chromatographic column and the fraction containing pure CCS-D2 were collected. The buffer was then exchanged in anaerobic conditions to remove dissolved oxygen.

NMR experiments. In-cell NMR spectra were collected at 308 K at a 950 MHz Bruker Avance III spectrometer equipped with a TCI CryoProbe. For each cell sample, a 2D [¹H-¹⁵N] SOFAST-HMQC[23] spectrum was recorded with 64 scans, 2048 points, 128 increments and a 0.3 s recycle delay (duration ~1 h). NMR spectra of the high glucose (Life Technologies) supplemented with L-glutamine (Life Technologies, 2 mM final concentration), antibiotics (penicillin and streptomycin, Life Technologies, 100 U/ml final concentration) and 10% FBS (Gibco) in uncoated 75 cm² plastic flasks and incubated at 37°C, 5% CO₂ in a humidified atmosphere. Cells were transiently transfected with the pHsec plasmid containing the gene of interest using polyethylenimine (PEI, branched, average Mₐ 25 kDa, Sigma-Aldrich), as previously described[18]. For co-expression of SOD1 (both WT and mutants) and CCS-D2, cells were transfected with plasmids containing the constructs in different amounts and ratios. The final ratio was chosen in order to obtain similar expression levels of the two proteins; specifically a weight ratio of 1:1:2 (25 µg SOD1 WT or mutant DNA; 25 µg CCS-D2 DNA; 50 µg PEI per 75 cm² flask) was used. [U-[¹⁵N]-BioExpress6000 medium (Cambridge Isotope Laboratories) was used for in-cell NMR samples, supplemented with 2% FBS, antibiotics and 10µM of ZnSO₄.

Protein quantification. The expression levels of WT/mutant SOD1 and CCS-D2 were determined by Coomassie-stained SDS-PAGE (Supplementary Figure S6). Lysates from cell samples co-expressing SOD1 and CCS-D2 were run at increasing dilutions together with purified WT SOD1 and CCS-D2 as references.
Densitometry analysis was performed with ImageJ. The values reported in the main text reflect the protein concentrations calculated in the cell lysates, which correspond to the effective concentrations in the in-cell NMR samples (mean value ± S.E.M.).

**Data Availability.** All the data generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
Lu.B., Le.B. and E.L. conceived the work; Le.B. and E.L. designed the experiments; Le.B. cloned the genes, grew the cells, produced the in-cell NMR samples and purified the proteins; E.L. performed the in-cell and in vitro NMR experiments and analyzed the data; Lu.B., Le.B. and E.L. wrote the manuscript.

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