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SUPPLEMENTAL INFORMATION

The importance of Zn(II) binding by the human copper metallochaperone for Cu,Zn-superoxide dismutase

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1. MATERIALS AND METHODS

Cloning of Human Cu,Zn-superoxide dismutase

The gene coding for human Cu,Zn-superoxide dismutase (SOD1) was cloned from a pCI-neo construct[1] using the primers 5'- GAA**CATATG**GCGACGAAGGCCGTGTGCG-3' (forward) and 5'-GAA**GAATTC**TTATTGGGCGATCCCAATTACACC-3' (reverse) containing Nde1 and EcoR1 restriction sites (in bold, stop codon underlined). The resulting PCR product was ligated into p-GEMT and both strands of the gene were sequenced. The Nde1/EcoR1 fragment was subsequently cloned into pET29a to give pET29a_SOD1.

Expression and purification of proteins

The human copper metallochaperone for SOD1 (CCS) was expressed and purified as described previously.[2] For human SOD1, *E. coli* BL21 (DE3) transformed with pET29a_SOD1 was grown in LB media, containing 50 µg/mL kanamycin and 100 µM $Zn(SO_4)$, at 37 ^oC until an OD_{600} of 0.6-0.8. Protein expression was induced by addition of 1 mM isopropyl *β*-D-thiogalactopyranoside. Cells were incubated for a further 6 h before harvesting, 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 MilliQ water (Millipore) prior to being loaded onto a DEAE Sepharose FF column (~40 mL, GE Healthcare) and eluted with a linear NaCl (0-300 mM) gradient in 5 mM Tris pH 7.5 containing 1 mM EDTA and 1 mM DTT. Fractions containing SOD1, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were combined and exchanged via ultrafiltration (Amicon stirred cell, 10 kDa molecular mass cut-off membrane) into 20 mM Tris pH 7.5 plus 200 mM NaCl and 1 mM DTT. The final purification step was performed on either a Superdex 75 16/60 or a 10/300 GL column (GE Healthcare) in 20 mM Tris pH 7.5 containing 200 mM NaCl and 1 mM DTT. SOD1 eluted from both columns as a single peak (~11.5 mL corresponding to ~32 kDa, i.e. dimer, on the 10/300 GL column) and the fractions containing pure protein (≥ 90 %), as judged by SDS-PAGE, were combined. Matrix assisted laser desorption ionization time-of-flight mass spectrometry of SOD1 gave peaks of equal intensity at 15963 and 15796 Da, with the latter corresponding to the protein without the N-terminal Met introduced during cloning (calculated mass of 15804.6 Da). Atomic absorption spectroscopy (AAS) was used to determine the copper and zinc content of both CCS and SOD1 as described previously.[3] SOD1 was purified with 1 equivalent of Zn(II) and no copper (E,Zn-SOD1).

Preparation of Zn(II)-free CCS

Zn(II)-free CCS was prepared using a modified version of a procedure for SOD1 demetallation.[4] CCS (70-140 μM) in 5 mL of 20 mM Tris pH 7.5 containing 1 mM DTT was dialyzed at 4 ^{o}C against 500 mL of 50 mM sodium acetate pH 4.5 plus 50 mM EDTA. The dialysis buffer was changed after 3, 6 and 18-22 hours. The protein was dialysed into 20 mM 4-(2-hydroxyethyl)piperazine-1-ethansulfonic acid (Hepes) pH 7.5 containing 200 mM NaCl and 1 mM DTT (3 changes of buffer and ~ 3 hours per exchange). The protein was concentrated (Amicon ultra 4 centrifugal filter units, 10 kDa molecular weight cut-off) and applied to a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in the same buffer. The fractions corresponding to Zn(II)-free CCS (verified by AAS) that eluted as a monomer (the major peak was at ~10.9 mL corresponding to ~40 kDa even at loaded protein concentrations of 700 μM) were collected and combined.

Preparation of protein samples

Zn(II)-free CCS (~ 100 μM) was reduced by incubation with 10 mM DTT in 20 mM Hepes pH 7.5 plus 200 mM NaCl for 2-3 h. CCS (containing Zn(II)) and E,Zn-SOD1 (200-800 μM) were reduced with 10 mM DTT for 2-3 and 12 h, respectively. Protein samples were incubated in an anaerobic chamber (Belle Technology, < 2 ppm O_2) and desalted and exchanged into 20 mM Hepes pH 7.5 containing 200 mM NaCl using a PD10 column (GE Healthcare). The CCS concentration was routinely determined using the absorbance at 280 nm (12490 M^{-1} cm^{-1})[5] and occasionally with a Bradford assay (Coomassie Plus protein assay kit, Thermo Scientific).[2] The concentration of SOD1 was measured using the absorbance at 280 nm (5500 $M^{-1}cm^{-1}$).[5] The number of free thiols for DTT treated Zn(II)-free CCS and SOD1 were quantified in anaerobic quartz cuvettes (Hellma) with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) in Ellman's buffer (100 mM potassium phosphate pH 8.0 containing 1 mM EDTA) by UV/Visible (UV/Vis) spectroscopy (Perkin Elmer λ35 spectrophotometer) using a molar absorption coefficient of 14150 M^{-1} cm^{-1} at 412 nm for the TNB^{2-} product.[6] After reduction, E,Zn-SOD1 routinely gave ~2 thiols per monomer (reaction with DTNB complete in minutes) after overnight incubation with DTT (~1 thiol prior to this treatment for protein purified in the presence of 1 mM DTT), which are assumed to belong to Cys57 and Cys147 that form the essential intra-molecular disulfide (Cys6 of SOD1 does not react with DTNB whilst Cys111 reacts very slowly[7,8]).

Cu(I) binding to CCS

A Cu(I) solution (50 mM $[Cu(CH_3CN)_4]PF_6$ in 100 % acetonitrile) was diluted to ~1 mM in 20 mM Hepes plus 200 mM NaCl at pH 7.5. Cu(I) was added either to CCS in open vessels in the anaerobic chamber to prepare Cu(I)-bound samples for analytical gel filtration or in a sealed anaerobic quartz cuvette using a gastight syringe (Hamilton) for titrations monitored by UV/Vis spectroscopy. Copper concentrations were determined either by AAS[3] or using the chromophoric Cu(I) ligands bathocuproine disulfonate (BCS) or bicinchoninic acid (BCA) with extinction coefficients of 12500 and 7700 M^{-1} cm^{-1} at 483 and 562 nm, respectively for $[Cu(BCS)_2]^{3-}$ and $[Cu(BCA)_2]^{3-}$.[9-12] To determine the stoichiometry of tight binding, Cu(I) was titrated into Zn(II)-free CCS (10 μM) in the presence of BCA (500 μM) in 20 mM Hepes pH 7.5 containing 200 mM NaCl.

The affinity (K_b value) of the first Cu(I) bound to Zn(II)-free CCS was determined from UV/Vis-monitored titrations of BCS into Zn(II)-free CCS (10 μM) plus 5 μM Cu(I) in 20 mM Hepes containing 200 mM NaCl at pH 7.5. The data were fit to a 1:1 Cu(I):protein binding model (Equation I) with Origin (Microcal) to determine the Cu(I) affinity (using a β value of 6.3×10^{19} M^{-2} for formation of the $[Cu(BCS)_2]^{3-}$ complex)[9] as described previously.[10,11]

$$[L]=2[CuL_2]+\sqrt{\frac{K_b([P]-[Cu]+[CuL_2])[CuL_2]}{([Cu]-[CuL_2])\beta}} \qquad (I)$$

In the above equation [L], [P] and [Cu] represent the total concentrations of BCS, protein and Cu(I) respectively.

Analytical gel filtration chromatography and circular dichroism spectroscopy

Analytical gel filtration chromatography was performed on Zn(II)-free CCS and Zn(II)-CCS (10 and 50 μM). The influence of adding different amounts of Cu(I) (*vide infra*) to Zn(II)-free CCS was also analysed by gel filtration chromatography. Zn(II)-free CCS and Zn(II)-CCS were incubated with an equimolar amount of E,Zn-SOD1 (10 and 50 μM) for ~5 minutes under anaerobic conditions and the mixtures were investigated by gel filtration chromatography. Samples (150 μL injection volumes) were analysed on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in nitrogen purged 20 mM Hepes containing 200 mM NaCl at pH 7.5 at a flow rate of 0.8 mL/min with the absorbance monitored at 280 nm. Oxygen was removed from the gel filtration buffer, which was maintained under a nitrogen atmosphere during

chromatography. Apparent molecular weights were calculated from elution volumes using a low molecular weight calibration kit (GE Healthcare) containing aprotonin (6.5 kDa), ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (44 kDa) and conalbumin (75 kDa). Far-UV CD spectra (190-250 nm) of Zn(II)-free CCS and Zn(II)-CCS (0.5 mg/mL) were recorded on a JASCO J-810 spectrometer in 20 mM Hepes pH 7.5 containing 200 mM NaCl at 20 ^{o}C.

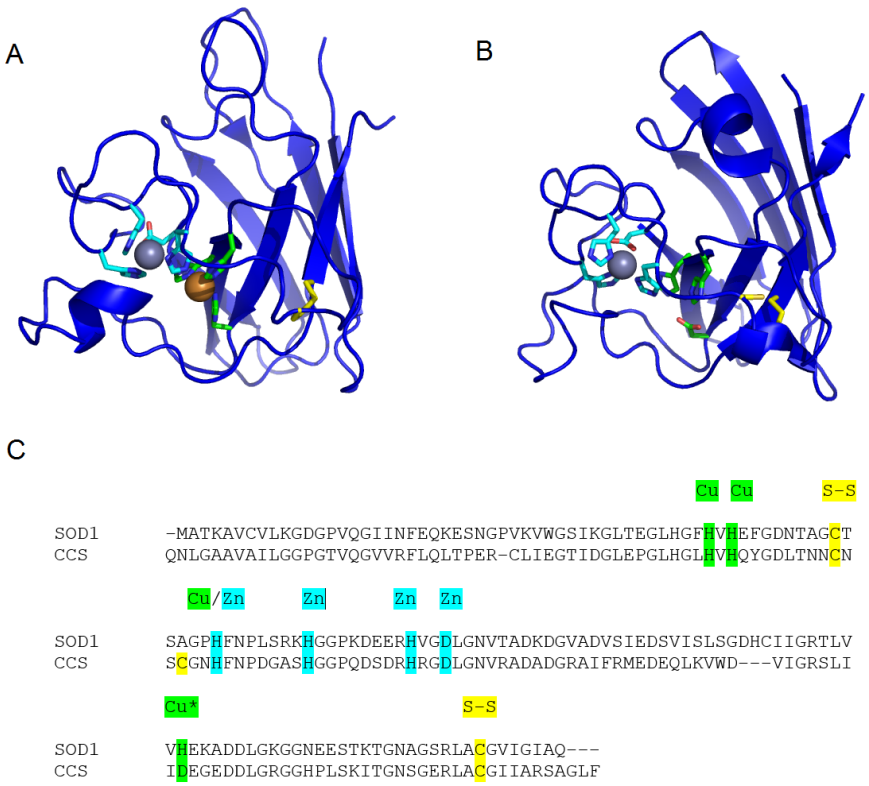

Figure S1. Crystal structures of (A) human SOD1 (1SPD)[13] and (B) domain 2 of human CCS (1DO5),[14] with one monomer shown in each case. An alignment (using ClustalW) of the amino-acid sequences is shown in (C). The zinc and copper ions are depicted as grey and gold spheres respectively, and the coordinating amino acids and the Cys residues of the disulfide (Cys57 and Cys146 in SOD1 and Cys141 and Cys227 in CCS) and Cys144 (CCS only) are shown as stick representations. Zinc ligands are cyan, while the copper-coordinating residues in SOD1, and the corresponding amino acids in CCS, are green.

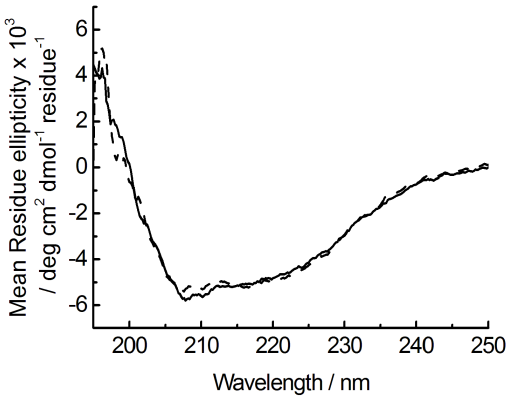

Figure S2. Far-UV CD spectra of Zn(II)-CCS (solid line) and Zn(II)-free CCS (dashed line) (0.5 mg/mL) in 20 mM Hepes pH 7.5 containing 200 mM NaCl.

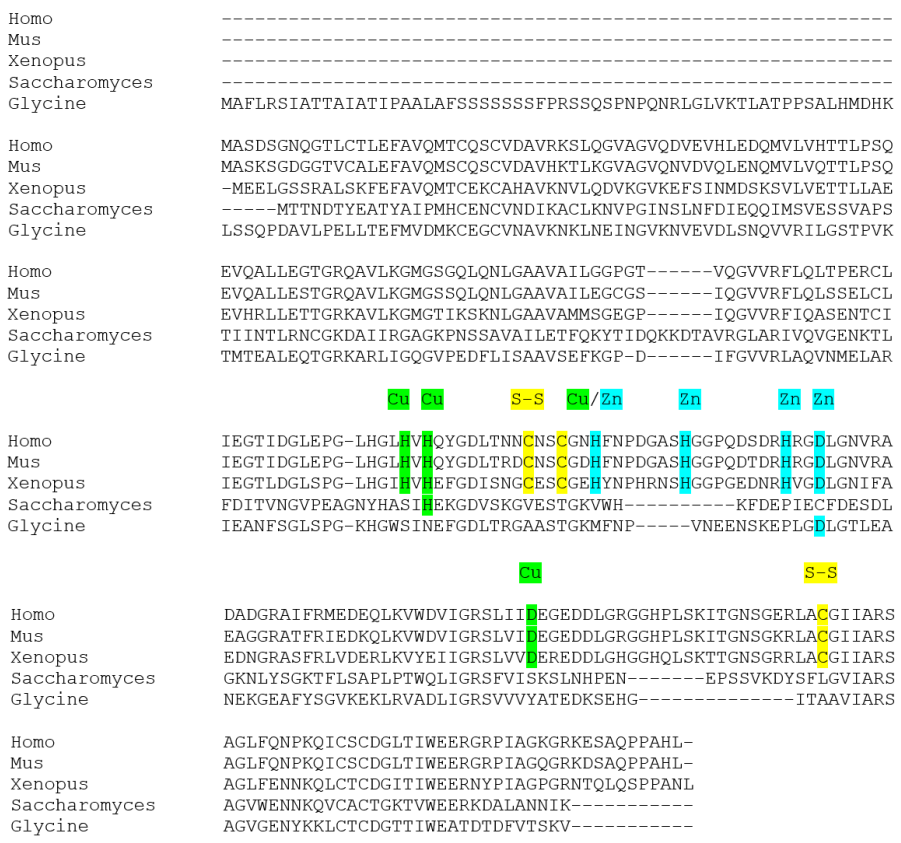

Figure S3. Sequence alignment (using ClustalW) of human (*Homo sapiens*) CCS with the proteins from mouse (*Mus musculus*), *Xenopus laevis* (African clawed frog) *Saccharomyces cerevisae* and *Glycine max* (soybean). Residues involved in binding zinc are highlighted blue and the amino acids corresponding to those that bind copper in human SOD1 are highlighted green. The Cys residues that form the disulfide in human CCS (Cys141 and Cys227) and Cys 144, and corresponding Cys residues when present in homologues, are highlighted yellow. Domain 2 of CCS from *M. musculus* and *X. laevis* also appear to have the zinc site and the disulfide that are present in the human protein (see Figure S1), whereas these are absent in *S. cerevisae* and *G. max*.

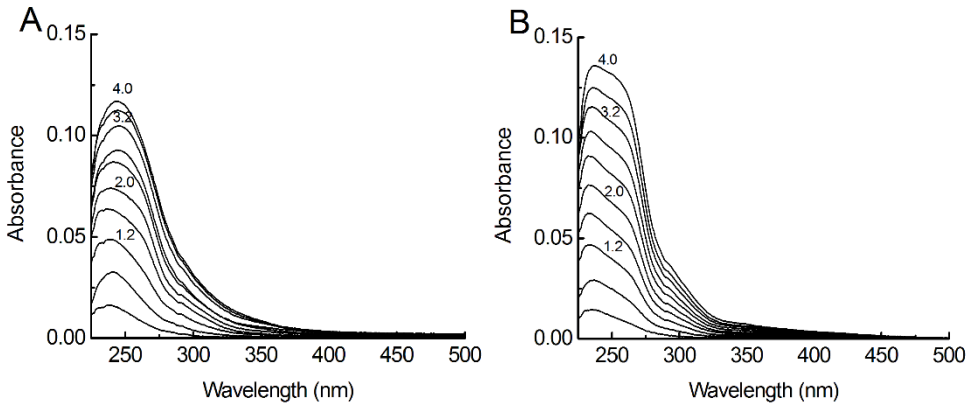

Figure S4. UV/Vis difference spectra of Zn(II)-CCS (A) and Zn(II)-free CCS (B) (both 10 μM) titrated with Cu(I) (0.4 molar equivalents per addition) in 20 mM Hepes pH 7.5 containing 200 mM NaCl.

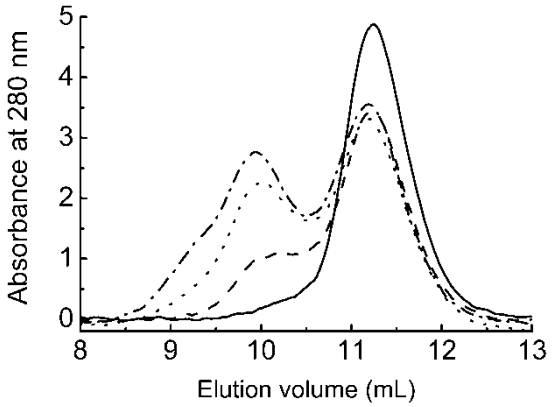

Figure S5. Analytical gel filtration chromatograms of Zn(II)-free CCS (solid line) and for samples to which 1, 2 and 3 equivalents of Cu(I) have been added (dash, dot and dash-dot lines respectively). All samples were loaded at a protein concentration of 10 μM and the chromatography was performed in degassed and nitrogen-purged 20 mM Hepes pH 7.5 containing 200 mM NaCl. The addition of Cu(I) results in dimer formation, with the second equivalent giving the largest increase in the dimer:monomer ratio.

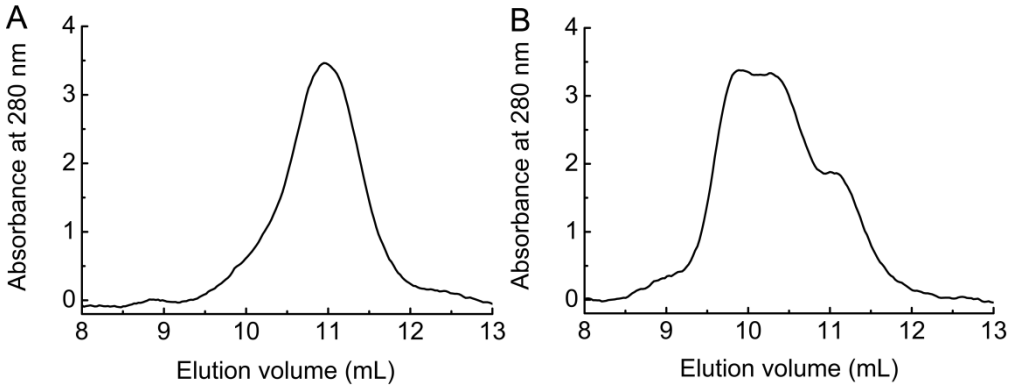

Figure S6. Analytical gel-filtration chromatograms of mixtures of Zn(II)-free CCS plus E,Zn-SOD1 (A) and Zn(II)-CCS plus E,Zn-SOD1 (B) at 10 μM in degassed and nitrogen-purged 20 mM Hepes pH 7.5 containing 200 mM NaCl. In (A) monomeric Zn(II)-free CCS and dimeric E,Zn-SOD1 co-elute due to very similar molecular weights (Table S1), whereas in (B) the hetero-complex is present (elution volume of 10.4 mL), along with some dimeric Zn(II)-CCS at a lower elution volume (higher apparent molecular weight, see Table S1). The relative amount of hetero-complex compared to Zn(II)-CCS and E,Zn-SOD1 is greater in the experiment performed at 50 (Figure 3A) compared to 10 (A) μM

Table S1 Elution volumes and corresponding calculated apparent molecular weights for Zn(II)-CCS and Zn(II)-free CCS, E,Zn-SOD1 and the mixtures indicated, determined from the analytical gel filtration chromatograms shown in Figure 1 and Figure S6 (except for E,Zn-SOD1)[a]

Protein	Elution volume (mL)	Apparent molecular weights (kDa)
Zn(II)-CCS	9.7	64 (± 2)
Zn(II)-free CCS	10.8	41 (± 1)
E,Zn-SOD1	11.4	33 (± 1)
Zn(II)-CCS + E,Zn-SOD1	9.7	64 (± 4)
	10.4	48 (± 5)
	11.0	38 (± 4)
Zn(II)-free CCS + E,Zn-SOD1	11.0	38 (± 4)

[a] The actual molecular weights of a CCS monomer is 28.9 kDa (57.8 kDa for the dimer) whilst that of a SOD1 dimer is 31.8 kDa.

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