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ARTICLE TYPE

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

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The human copper metallochaperone (CCS) for Cu,Zn-superoxide dismutase (SOD1) has a similar Zn(II) site as that in SOD1. Dimeric CCS converts to a monomer in the reduced Zn(II)-depleted form and cannot interact with SOD1. This form of CCS may be fibrillogenic and disease causing, as is the case for demetallated and reduced monomeric SOD1.

Human Cu,Zn-superoxide dismutase (SOD1) is responsible for the removal of a reactive oxygen species (ROS); molecules linked to numerous diseases and ageing.[1] Mutations in SOD1 can lead to misfolding and aggregation resulting in the neurodegenerative disorder familial amyotrophic lateral sclerosis.[2] Furthermore, SOD1 has been found to play a vital role in nutrient sensing giving it potential importance to many cancers.[3] SOD1 is the most abundant cytosolic protein with an essential disulphide, between Cys57 and Cys146, and is the only enzyme known to acquire copper in this location. Copper incorporation and disulfide bond formation, key steps in the activation of SOD1,[4,5] can occur via two pathways, with the dominant route utilising CCS, a copper metallochaperone.[6] CCS has three domains with copper delivery and disulfide bond formation performed by domains 1 and 3 (D1 and D3), while the largest domain 2 (D2) facilitates the interaction with SOD1.[7-14] D2 of human CCS has high sequence (47%) and structural homology to SOD1 (Figure S1, see *Supplemental Information*), with an identical Zn(II) site in the same location, and a disulfide between Cys141 and Cys227 corresponding to that between Cys57 and Cys146 in SOD1.[15] Numerous studies have investigated the importance of zinc and copper binding, as well as the disulfide, for SOD1.[16-21] Removal of both metals from the active site results in structural disorder,[20] and in the absence of Zn(II) and the disulfide, dimeric SOD1 converts to a monomer,[16,17,19] a form more prone to aggregation.[21] The acquisition of Zn(II) by SOD1 is thought to be the first step in the activation process,[5,14] occurs in the absence of CCS,[22] and does not require a metallochaperone, even though Zn(II) binding at the copper site, which can occur *in vitro*, is precluded.[22,23]

Despite the proven importance of Zn(II) for SOD1, the role of the homologous Zn(II) site in D2 of human CCS has not been studied *in vitro*. CCS, like mature SOD1, is dimeric,[10,13] with a hetero-dimeric complex involved in activation.[9,14] To assess the function of the Zn(II) site of CCS we have produced fully apo-protein, i.e. with no Cu(I) or Zn(II) bound, using a procedure including the reductant dithiothreitol (DTT) (see *Supplemental Information*). The overall secondary structure of CCS is not dramatically altered by Zn(II) removal (Figure S2, see *Supplemental Information*). However, this form of CCS is predominantly monomeric (Figure 1 and Table S1, see *Supplemental Information*), and has approximately two more free thiols than the Zn(II)-protein (7.3 ± 1.1 compared to 5.1 ± 0.6 per monomer). These extra thiols most likely arise from Cys141 and Cys227, as the Zn(II)-free form of SOD1 with the Cys57-Cys146 disulfide reduced is also monomeric, and disulfide formation in the absence of Zn(II) results in dimeric SOD1.[16] The Cys141-Cys227 disulfide is more readily cleaved by reductants in Zn(II)-free CCS, which is also the case for the corresponding disulfide in Zn(II)-free SOD1,[17,18] although its reduction potential is unaltered.[24] Our observation that Zn(II) removal and D2 disulfide cleavage greatly favours the monomeric form of human CCS is also consistent with data showing that *Saccharomyces cerevisiae* CCS, which lacks the Zn(II) site and the disulfide in D2 (Figure S3, see *Supplemental Information*), is monomeric.[13,25] Incubation of Zn(II)-free CCS with Zn(II) under anaerobic conditions results in relatively quick (> 50% in 5 min) dimer re-formation (unaffected by the presence of DTT in the gel-filtration buffer). Therefore, Zn(II)-binding alone would appear to stabilise the dimeric form of CCS, as is the case for SOD1.[16]

We have investigated how the removal of Zn(II) from human CCS influences its ability to bind Cu(I). A comparison of the

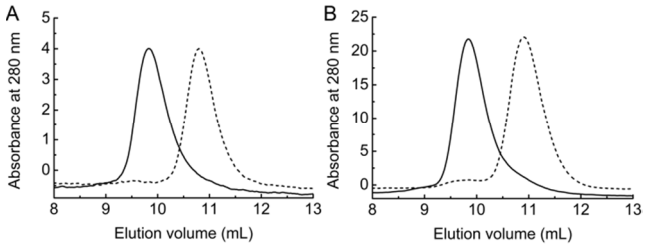

Fig. 1 Analytical gel filtration chromatograms of Zn(II)-CCS (solid line) and Zn(II)-free CCS (dashed line) at loaded concentrations of 10 (A) and 50 (B) μM in degassed and nitrogen-purged 20 mM Hepes pH 7.5 containing 200 mM NaCl (the apparent molecular weights calculated from elution volumes are listed in Table S1, see *Supplemental Information*).

titration of Cu(I) into Zn(II)-free CCS in the presence of the high affinity Cu(I) ligand bicinchoninic acid (BCA), with that of the Zn(II)-protein, is shown in Figure 2A. The Zn(II)-free protein binds an additional equivalent of Cu(I) via Cys residues (Figure S4, see *Supplemental Information*) that out-competes BCA under the conditions used (Figure 2A). The Cu(I) affinity of the tightest site in Zn(II)-free CCS (Figure 2B) is almost identical to that of Zn(II)-bound protein ($(5.5 \pm 0.6) \times 10^{17}$ M^{-1} at pH 7.5), which we therefore assume binds to the D1 CXXC motif as in the Zn(II)-protein.[13,14] The D3 CXC site has a Cu(I) affinity at least an order of magnitude weaker than that in D1 (2.7×10^{16} M^{-1} at pH 7.5) in the Zn(II)-protein,[13] and the affinity of the weakest site in Zn(II)-free CCS appears to be very similar (Figure 2A), and is presumably also in D3.[13] The additional Cu(I) site most likely involves Cys141 and Cys227 of the reduced disulfide in D2 of Zn(II)-free CCS, and maybe also Cys144 that is 5.3 and 4.2 Å respectively from these residues (Figure S1, see *Supplemental Information*).[15] The binding of Cu(I) at this site is relatively tight (~5×10^{16} M^{-1} to 5×10^{17} M^{-1} at pH 7.5), and facilitates dimer formation (Figure S5). Cu(I) binding to these Cys residues must partially stabilise the structure of loop 4 on which Cys144 and the Zn(II) ligands are located and that contributes to the dimer interface.[15] This additional Cu(I) site would appear to have little physiological relevance, and Cu(I) binding here may be a reaction that needs to be prevented by maintaining low levels of weakly bound copper in a cell.[26] However, the Cys residues that form the corresponding disulfide in SOD1 have been suggested to be involved in the Cu(I) transfer mechanism from CCS.[27] Our studies indicate that Cu(I) binding at this site in SOD1 should also be relatively tight.

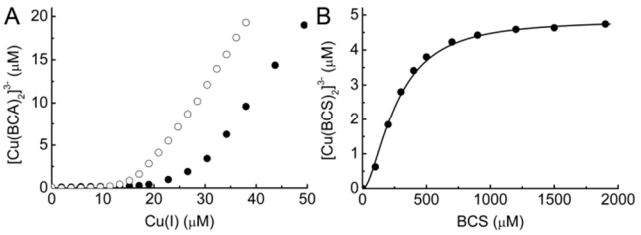

Fig. 2 (A) The titration of Cu(I) into Zn(II)-free CCS (solid circles)[13] and Zn(II)-CCS (open circles) (both 10 μM) in the presence of BCA (500 μM) in 20 mM Hepes pH 7.5 containing 200 mM NaCl. (B) The titration of BCS into Zn(II)-free CCS (10 μM) loaded with 0.5 equivalents of Cu(I) in the same buffer. The line shows a fit of the data to equation I (see *Supplemental Information*) giving a Cu(I) affinity (K_b value) of $(5.2 \pm 0.4) \times 10^{17}$ M^{-1}.

To test if Zn(II) binding by CCS influences complex formation with copper-free SOD1 (E,Zn-SOD1), we have undertaken preliminary gel-filtration studies (proteins were reduced with DTT, desalted, mixed under anaerobic conditions, and analysed by gel-filtration chromatography using degassed and nitrogen-purged buffer, see *Supplemental Information*). Mixtures of Zn(II)-free CCS and E,Zn-SOD1 show no sign of the hetero-complex, even at protein concentrations of 50 μM (when loaded onto the column, Figure 3A, Figure S6A and Table S1, see *Supplemental Information*). Chromatograms for Zn(II)-CCS mixed with E,Zn-SOD1 are consistent with relatively tight complex formation (Figure 3B, Figure S6B and Table S1, see *Supplemental Information*). Very similar data were obtained when the gel-filtration chromatography was performed in the presence of 1 mM DTT. The removal of Zn(II) from CCS therefore not only destabilises the homo-dimer but also weakens complex formation with SOD1.

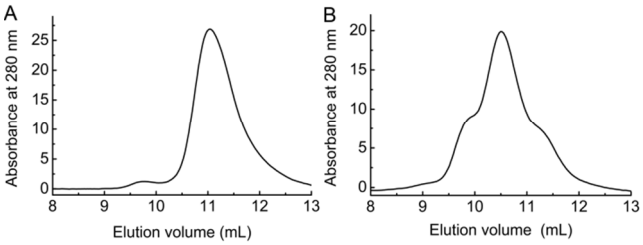

Fig. 3 Analytical gel-filtration chromatograms of a mixture of Zn(II)-free CCS plus E,Zn-SOD1 (A) and Zn(II)-CCS plus E,Zn-SOD1 (B) with both proteins at 50 μM and the chromatography performed in degassed and nitrogen-purged 20 mM Hepes pH 7.5 containing 200 mM NaCl. In (A) monomeric Zn(II)-free CCS and dimeric E,Zn-SOD1 co-elute due to very similar molecular weights (Table S1, see *Supplemental Information*), 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, Table S1, see *Supplemental Information*).

It has been shown that removal of single Zn(II) ligands in the H147A and H164A mutants results in human CCS being unable to activate SOD1 *in vivo*.[28] Although the total levels of CCS are unaffected, the soluble forms of these mutants decrease dramatically.[28] As we have shown, in the absence of Zn(II) and the Cys141-Cys227 disulfide human CCS will exist as a monomer that cannot interact with SOD1. This form of CCS may be fibrillogenic and disease causing, as is the case for demetallated monomeric SOD1 that also lacks the disulfide.[18,21] Additionally, some SOD1 and CCS localise to the inter-membrane space (IMS) of mitochondria helping to protect against oxidative damage.[29] The import mechanism for human CCS into the IMS differs to that of SOD1, yet immature protein (reduced and unfolded), which will not have any Zn(II) bound, is the preferred form of both proteins for uptake.[30,31] Disulfide formation during uptake helps trap CCS in the IMS.[31] Based on our observations the import of human CCS into mitochondria must also be dependent upon Zn(II) binding.

Conclusions

In these studies we show that Zn(II) binding in D2 of human CCS stabilises both its quaternary structure and the interaction with SOD1. The Zn(II)-free monomeric form of CCS has an additional tight binding site for Cu(I), presumably using the Cys residues from the disulfide, which is more readily reduced in this form of the protein. Zn(II) binding and disulfide bond formation in D2 of CCS are required prior to SOD1 activation and must also play a role in the protein's uptake into the IMS. These results further highlight the importance of Zn(II) insertion into the correct sites of proteins that are essential for maintaining health. The cellular mechanisms which ensure that both human CCS and SOD1 bind Zn(II) at the correct site remain to be discovered.

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Notes and references

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† Electronic Supplementary Information (ESI) available: materials and methods, figures comparing the structures and sequences of human SOD1 and CCS, the sequences of CCS from different sources, far-UV CD spectra, UV/Vis mointored Cu(I) titrations and a figure and table showing analytical gel filtration data. See DOI: 10.1039/b000000x/

1 K. J. Barnham, C. L. Masters and A. I. Bush, *Nat. Rev. Drug Discov.*, 2004, **3**, 205.
2 D. W. Cleveland and J. D. Rothstein, *Nat. Rev. Neurosci.*, 2001, **2**, 806.
3 A. R. Reddi and V. C. Culotta, *Cell*, 2013, **152**, 224.
4 N. M. Brown, A. S. Torres, P. E. Doan and T. V. O'Halloran, *Proc. Natl. Acad. Sci. U.S.A.*, 2004, **101**, 5518.
5 Y. Furukawa, A. S. Torres and T. V. O'Halloran, *EMBO J.*, 2004, **23**, 2872.
6 M. C. Carroll J. B. Girouard, J. L. Ulloa, J. R. Subramaniam, P. C.Wong, J. Valentine and V. C. Culotta, *Proc. Natl. Acad. Sci. U.S.A.*, 2004, **101**, 5964.
7 P. J. Schmidt, T. D. Rae, R. A. Pufahl, T. Hamma, J. Strain, T. V. O'Halloran and V. C. Culotta, *J. Biol. Chem*., 1999, **274**, 23719.
8 A. L. Lamb, A. K. Wernimont, R. A. Pufahl, V. C. Culotta, T. V. O'Halloran and A. C. Rosenzweig, *Nat. Struc. Biol*., 1999, **6**, 724.
9 A. L. Lamb, A. S. Torres, T. V. O'Halloran and A. C. Rosenzweig, *Nat. Struc. Biol*., 2001, **8**, 751.
10 T. D. Rae, A. S. Torres, R. A. Pufahl and T. V. O'Halloran, *J. Biol. Chem*., 2001, **276**, 5166.
11 A. L. Caruano-Yzermans, T. B. Bartnikas and J. D. Gitlin, *J. Biol. Chem*., 2006, **281**, 13581.
12 K. Kirby, L. T. Jensen, J. Binnington, A. J. Hilliker, J. Ulloa, V. C. Culotta and J. P. Phillips, *J. Biol. Chem.*, 2008, **283**, 35393.
13 S. Allen, A. Badarau and C. Dennison, *Biochemistry*, 2012, **51**, 1439.
14 L. Banci, I. Bertini, F. Cantini, T. Kozyreva, C. Massagni, P. Palumaa, J. T. Rubino and K. Zovo, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, **109**, 13555.
15 A. L. Lamb, A. K. Wernimont, R. A. Pufahl, T. V. O'Halloran and A. C. Rosenzweig, *Biochemistry*, 2000, **39**, 1589.
16 F. Arnesano, L. Banci, I. Bertini, M. Martinelli, Y. Furukawa and T. V. O'Halloran, *J. Biol. Chem.*, 2004, **279**, 47998.
17 P. A. Doucette, L. J. Whitson, X. Cao, V. Schirf, B. Demeler, J. S. Valentine, J. C. Hansen and P. J. Hart, *J. Biol. Chem.* 2004, **279**, 54558.
18 B. R. Roberts, J. A. Tainer, E. D. Getzoff, D. A. Malencik, S. R. Anderson, V. C. Bomben, K. R. Meyers, P. A. Karplus and J. S. Beckman, *J. Mol. Biol.* 2007, **373**, 877.
19 S. Z. Potter, H. Zhu, B. F. Shaw, J. A. Rodriguez, P. A. Doucette, S. H. Sohn, A. Durazo, K. F. Faull, E. B. Gralla, A. M. Nersissian and J. S. Valentine, *J. Am. Chem. Soc.*, 2007, **129**, 4575.
20 L. Banci, I. Bertini, M. Boca, V. Calderone, F. Cantini, S. Girotto and M. Vieru, *Proc. Natl. Acad. Sci. U.S.A.*, 2009, **106**, 6980.
21 L. Banci, I. Bertini, O. Blazevits, F. Cantini, M. Lelli, C. Luchinat, J. Mao and M. Vieru, *J. Am. Chem. Soc*., 2011, **133**, 345.
22 L. Banci, L. Barbieri, I. Bertini, E. Luchinat, E. Secci, Y. Zhao and A. R. Aricescu, *Nat. Chem. Biol.*, 2013, **9**, 297.
23 L. Banci, L. Barbieri, I. Bertini, F. Cantini and E. Luchinat, *PloS ONE*, 2011, **6**, 23561.
24 S. D. Bouldin, M. A. Darch, P. J. Hart and C. E. Outten, *Biochem. J.* 2012, **446**, 59.
25 A. S. Torres, V. Petri, T. D. Rae and T. V. O'Halloran, *J. Biol. Chem.* 2001, **276**, 38410.
26 T. D. Rae, P. J. Schmidt, R. A. Pufahl, V. C. Culotta and T. V. O'Halloran, *Science*, 1999, **284**, 805.
27 L. Banci, F. Cantini, T. Kozyreva and J. T. Rubino, *ChemBioChem*, 2013, **14**, 1839.
28 T. Endo, T. Fujii, K. Sato, N. Taniguchi and J. Fujii, *Biochem. Biophys. Res. Commun*., 2000, **276**, 999.
29 L. A. Strutz, K. Diekert, L. T. Jensen, R. Lill and V. C. Culotta, *J. Biol. Chem.* **2001**, 276, 38084.
30 L. S. Field, Y. Furukawa, T. V. O'Halloran and V. C. Culotta, *J. Biol. Chem.* **2003**, 278, 28052.
31 Y. Suzuki, M. Ali, M. Fischer and J. Riemer, *Nat. Commun*. 2013, **4**, 2430.