Supporting information on determination of Cu^I binding stoichiometry and affinity for Atox1 (Hah1) protein

Protein expression, isolation and characterisation. The full length Atox1 gene was cloned into the expression vector pET20b with restriction sites *Nde*I and *Bam*HI and the expression plasmid was transformed into *E. coli* strain BL21(DE3)pLysS cells for protein expression. After the cells were grown to an OD_{600} ~ 1 at 37 °C in 2YT medium, expression of the Atox1 protein was induced with IPTG (0.4 mM) at ~ 30 °C for 4 h. The Atox1 protein was purified by a gradient elution (0 – 0.3 M NaCl) from a cation-exchange CM-52 column equilibrated in an acetate buffer (20 mM, pH 5.4; 5 mM β-mercaptoethanol), followed by a Superdex-75 FPLC gel-filtration column in KPi buffer (20 mM, pH 7.0; 150 mM NaCl; 0.5 mM Tcep). The purified protein was confirmed by SDS-PAGE (purity > 95%) and by ESI-MS (two components at 7270.5 and 7401.7 Da, corresponding to Atxo1 without and with the first methionine residue, respectively, as observed previously: Ralle, M.; Lutsenko, S. and Blackburn, N. J. *J. Biol. Chem.* 2003, **278**, 23163-23170).

The purified protein was reduced fully by incubation with Dtt (5 mM) overnight in an anaerobic glove-box and separated from Dtt with a Bio-Del P-6 DG gel desalting column (Bio-Rad) in the glove-box. Thiol assay with Ellman reagent confirmed the expected thiol content (3 equiv per Atox1 molecule), on the basis of protein concentration estimated from the absorbance at 280 nm. The extinct coefficient (ε, 2,980 M^{-1} cm^{-1}; see section 4.4.3) was calculated from the primary protein sequence.

Estimation of Cu^I binding stoichiometry and affinity. The Cu^I binding stoichiometry was estimated by quantitative removal of Cu^I from the complex $[Cu^I(Bca)_2]^{3-}$ (eq 1) and the affinity

was estimated by competition for Cu^{I} with ligand Bcs (eq 2). The validities of both equations were confirmed by variation of ligand (Bca or Bcs) concentration, as given in Table S1.

$$\text{Atox1} + [\text{Cu}^{\text{I}}(\text{Bca})_2]^{3-} \longrightarrow \text{Cu}^{\text{I}}\text{-Atox1} + 2\,\text{Bca}^{2-} \quad \text{(S1)}$$

$$\text{Atox1} + [\text{Cu}^{\text{I}}(\text{Bcs})_2]^{3-} \rightleftharpoons \text{Cu}^{\text{I}}\text{-Atox1} + 2\,\text{Bcs}^{2-} \quad \text{(S2)}$$

Table S1. Estimation of Cu^{I}-binding stoichiometry for Atox1 according to eq S1 [a]

$[\text{Atox1}]_{total}$ (μM)	$[\text{Bca}]_{total}$ (μM)	$[\text{Cu}^{\text{I}}]_{total}$ (μM)	A_{562}	$[\text{Cu(Bcs)}_2]^{3-}$ (μM)	$[\text{Cu}^{\text{I}}\text{P}]/[\text{P}]_{total}$ (θ)
0	100	36.2	0.286	36.2	-
10	100	36.2	0.211	26.7	0.94
15	100	36.2	0.157	19.9	1.09
20	100	36.2	0.139	17.6	0.93
26	100	36.2	0.090	11.4	0.95
10	200	36.2	0.214	27.1	0.91
20	200	36.2	0.116	14.7	1.08
26	200	36.2	0.082	10.4	1.0
15	500	36.2	0.154	19.5	1.1

[a] In KPi buffer (25 mM, pH 7.0; NaCl 100 mM) under anaerobic conditions.

It can be seen from Table S1 that Atox1 can bind one eq Cu^{I} /Atox1 molecule and that the Cu^{I} occupancy on Atox1 remains unchanged with increasing concentrations of either Atox1 or/and ligand Bca. Consequently, within the concentration range 100 – 500 μM, the Cu^{I} affinity of Bca is too weak to compete for Cu^{I} with the protein.

Table S2. Estimation of Cu^I-binding affinity (K_D) for Atox1 (P) according to eq S2 [a]

$[Bcs]_{total}$ (μM)	$[Cu^I]_{total}$ (μM)	$[P]_{total}$ (μM)	A_{483}	$[Cu(Bcs)_2]^{3-}$ (μM)	$[Cu^IP]$ (μM)	$[Cu^IP]/[P]_{total}$ (θ)	$K_{D(CuP)}$ [b] (M)
200	36.5	0	0.475	36.5	-	-	
200	36.5	10	0.367	28.2	8.3	0.83	Average from curve fitting 4.0×10^{-18}
200	36.5	20	0.280	21.5	15.0	0.75	
200	36.5	30	0.207	15.9	20.6	0.69	
200	36.5	40	0.158	12.1	24.4	0.61	
200	36.5	50	0.126	9.7	26.8	0.54	
500	34.8	0	0.453	34.8	-	-	
500	34.8	10	0.404	31.1	3.8	0.38	Average from curve fitting 3.8×10^{-18}
500	34.8	20	0.358	27.5	7.3	0.36	
500	34.8	40	0.295	22.7	12.2	0.31	
500	34.8	50	0.276	21.2	13.6	0.27	

[a] In KPi buffer (25 mM, pH 7.0; NaCl 100 mM) under anaerobic conditions.

[b] See Figure 13 for curve-fitting.

It can be seen from Table S2 that the Cu^I occupancy on Atox1 decreases with increasing concentrations of either the protein or Bcs ligands. Consequently, ligand Bcs competes for Cu^I effectively with Atox1 proteins.