Transfer of copper between *bis*(thiosemicarbazone) ligands and intracellular copper-binding proteins. Insights into mechanisms of copper uptake and hypoxia Selectivity

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Electronic Supplementary Information

Table S1. Electrochemical data [a]

Solvent [b]	pH [c]	Cu(Atsm)			Cu(Gtsm)			ΔE (V) [d]
		E_{Pc} (mV)	E_{Pa} (mV)	$E_{1/2}$ (mV)	E_{Pc} (mV)	E_{Pa} (mV)	$E_{1/2}$ (mV)	
Me_2SO /buffer (3:2 v/v)	6	- 590	-520	-555	- 390	ir [e]	-	- 0.2
	7	- 590	- 530	- 560	- 400	ir [e]	-	- 0.2
	8	-590	-525	- 555	- 395	ir [e]	-	- 0.2
	9	- 590	-530	- 560	- 400	ir [e]	-	- 0.2
Me_2SO	-	- 630	-570	- 600	- 480	-410	- 445	- 0.15
	-			- 590 [f]			- 430 [f]	- 0.15

[a] Potentials were referenced to Ag/AgCl in saturated KCl;

[b] Me_2SO containing 0.1 M $(Bu^n_4)NBF_4$ as electrolyte; aqueous buffer (50 mM) containing 0.1M NaCl as electrolyte;

[c] The buffers (50 mM) used were KPi (pH 6,7), Tris-Cl (pH 8) and Na-Ches (pH 9);

[d] Potential difference between Cu(Atsm) and Cu(Gtsm);

[e] Irreversible;

[f] From reference: Dearling, J. L.; Lewis, J. S.; Mullen, G. E.; Welch, M. J.; Blower, P. J. *J. Biol. Inorg. Chem.,* **2002,** *7*, 249-59.

Table S2 Estimation of $K_D(Cu^ILL)$ (L = Atsm or Gtsm) by ligand competition for Cu(I) with Bca.[a,b]

Ligand (LH_2)	$[Bca]_{tot}$ (μM)	$[LH_2]_{tot}$ (μM)	A_{562}	$[Cu^I(Bca)_2]^{3-}$ [c] (μM)	$[Cu^IL]$ [d] (μM)	$K_D(Cu^ILL)$ [e] (10^{-13} M)
none	100	0	0.322	40.0	0	-
$AtsmH_2$	0	50	0	0	40.0	-
	100	50	0.212	26.3	13.7	1.8
	100	100	0.192	23.9	16.1	2.7
	100	200	0.148	18.4	21.6	2.2
$GtsmH_2$	0	50	0.073	0	40.0	-
	100	50	0.222	23.9	16.1	1.1
	100	100	0.190	18.8	21.2	1.1
	100	200	0.162	14.3	25.7	1.1

[a] In KPi buffer (20 mM; pH 7) containing NaCl (100 mM), dithionite (1 mM) and DMSO (30%; v/v) under anaerobic conditions;

[b] Total Cu concentration in all equilibrium solutions was 40.0 μM;

[c] $[Cu^I(Bca)_2]^{3-}$ concentration was calculated as 40.0 x (A_{562}/0.322) and 40 x {(A_{562}– 0.073)/(0.322 – 0.073)} for competition with $AtsmH_2$ and $GtsmH_2$, respectively, since at 562 nm, the Cu(I) form has no absorption for $AtsmH_2$ but has weak absorption for $GtsmH_2$ (see Figures 6c, S1c);

[d] $[Cu^IL]$ = 40.0 - $[Cu^I(Bca)_2]^{3-}$ with assumptions that $[Cu^I]_{free} << [Cu^IL]$ and $[Cu^I]_{free} << [Cu^I(Bca)_2]^{3-}$;

[e] $K_D(Cu^ILL)$ was calculated according to following equation:

$$K_D(Cu^IP) = (1/\beta_2) \times (K_{ex})^{-1} = (1/\beta_2) \times \frac{[Cu^I(Bca)_2]\,[L]}{[Cu^IL]\,[Bca]^2}$$

where $\beta_2 = 1.7 \times 10^{17}$ for $[Cu^I(Bca)_2]^{3-}$ (see Table S3) and K_{ex} is the equilibrium constant for following exchange reaction:

$$[Cu^I(Bca)_2]^{3-} + L \Leftrightarrow Cu^I\text{-}L + 2\,Bca^{2-}$$

Determination of formation constant β_2 for $[Cu^I(Bca)_2]^{3-}$

If a competition for Cu(I) between a Cu(I)-binding protein P (or ligand L) and *both* Bca and Bcs can be induced in the *same* buffer conditions by variation of [Bca]/[Cu(I)] and [Bcs]/[Cu(I)] ratio, then we have following competitions:

$$[Cu^I(Bcs)_2]^{3-} + P \Leftrightarrow Cu^I\text{-}P + 2\,Bcs^{2-}$$

$$K_{ex} = \frac{[Cu^I\text{-}P]\,[Bcs]^2}{[Cu^I(Bcs)_2]\,[P]} = \{K_D(Cu^IP) \times \beta_2([Cu^I(Bcs)_2]^{3-})\}^{-1} \qquad (1)$$

Where $K_{D(Cu^IP)}$ is the dissociation constant of Cu^IP and $\beta_{2([Cu^I(Bcs)_2]^{3-})}$ is the formation constant of $[Cu^I(Bcs)_2]^{3-}$.

Similarly, $[Cu^I(Bca)_2]^{3-} + P \Leftrightarrow Cu^I\text{-}P + 2\,Bca^{2-}$

$$K_{ex}' = \frac{[Cu^I\text{-}P]\,[Bca]^2}{[Cu^I(Bca)_2]\,[P]} = \{K_D(Cu^IP) \times \beta_2([Cu^I(Bca)_2]^{3-})\}^{-1} \qquad (2)$$

In the *same* reaction buffer, $K_{D(Cu^IP)}$ in eqs (1) and (2) should be the same,

thus,
$$\frac{K_{ex}}{K_{ex}'} = \frac{K_D(Cu^IP) \times \beta_2([Cu^I(Bca)_2]^{3-})}{K_D(Cu^IP) \times \beta_2([Cu^I(Bcs)_2]^{3-})}$$

$$= \frac{\beta_2([Cu^I(Bca)_2]^{3-})}{\beta_2([Cu^I(Bcs)_2]^{3-})}$$

and
$$\beta_2([Cu^I(Bca)_2]^{3-}) = \frac{K_{ex}}{K_{ex}'} \times \beta_2([Cu^I(Bcs)_2]^{3-}) \qquad (3)$$

From the known β_2 for $[Cu^I(Bcs)_2]^{3-}$ ($10^{19.8}$)[1] and the experimental values K_{ex} and K_{ex}' for eqs (1) and (2), β_2 for $[Cu^I(Bca)_2]^{3-}$ can be calculated from (3).

Three proteins (Atx1[1], nA-PcoC,[2] C42S-rubredoxin[3]) were used to define the ratio of (K_{ex} / $K_{ex}^{'}$) in the same reaction buffer according to a previous approach.[1] These three proteins bind Cu(I) with very different affinity ($K_D = 10^{-13} - 10^{-18}$ M), but the ratios of (K_{ex} / $K_{ex}^{'}$) derived were very similar (Table S1), validating the reliability of the data obtained. Other experimental details and results are given in Tables S4 and S5.

References

1. Xiao, Z.; Loughlin, F.; George, G. N.; Howlett, G. J.; Wedd, A. G. *J. Am. Chem. Soc.,* **2004,** *126*, 3081-90.
2. Djoko, K. Y.; Xiao, Z.; Huffman, D. L.; Wedd, A. G. *Inorg. Chem.,* **2007,** *46*, 4560-8.
3. Xiao, Z.; Lavery, M. J.; Ayhan, M.; Scrofani, S. D. B.; Wilce, M. C. J.; Guss, J. M.; Tregloan, P. A.; George, G. N.; Wedd, A. G. *J. Am. Chem. Soc.,* **1998,** *120*, 4135-50.

Table S3. Exchange constants for eqs 1 and 2 and formation constant β_2 for $[Cu^I(Bca)_2]^{3-}$

apo-protein	K_{ex} (see eqs 1, 2) [a]		K_{ex}(Bcs) / K_{ex}(Bca)	β_2 ($[Cu^I(Bca)_2]^{3-}$) [b]
	Bca	Bcs		
nA-PcoC	6.6×10^{-5}	2.8×10^{-7}	4.2×10^{-3}	2.6×10^{17}
C42S-Rd	2.0	4.4×10^{-3}	2.2×10^{-3}	1.4×10^{17}
Atx1	4.5	7.8×10^{-3}	1.7×10^{-3}	1.1×10^{17}
Average			$2.7 (\pm 1.5) \times 10^{-3}$	$2(1) \times 10^{17}$

[a] Reactions were carried out in Na-Mops buffer (pH 7) and 100 mM NaCl;

[b] Calculated from eq (3) with known β_2 ($= 10^{19.8}$) for $[Cu^I(Bcs)_2]^{3-}$.

Table S4 Competition for Cu(I) between Bcs and *apo*-proteins nA-PcoC, C42S-Rd and Atx1 in Mops buffer (50 mM, pH 7) and 100 mM NaCl.

$[Bcs]_{total}$ (μM)	*Apo*-protein	$[P]_{total}$ (μM)	A_{483}	$[Cu(Bcs)_2]^{3-}$ (μM)	$[Cu^I\text{-}P]$ (μM)	K_{ex}	Average K_{ex}
70-500	none	0	0.395	30.4			
70	nA-PcoC	50	0.366	28.2	2.4	3.4×10^{-7}	2.8×10^{-7}
		100	0.361	27.8	2.8	2.2×10^{-7}	
400	C42S-Rd	20	0.278	21.4	9.0	4.9×10^{-3}	4.4×10^{-3}
		30	0.254	19.5	10.9	3.8×10^{-3}	
		40	0.217	16.7	13.7	4.2×10^{-3}	
		80	0.134	10.3	20.1	4.7×10^{-3}	
500	Atx1	10	0.329	25.3	4.9	7.5×10^{-3}	7.8×10^{-3}
		20	0.270	20.8	9.4	9.0×10^{-3}	
		30	0.236	18.2	12.0	7.9×10^{-3}	
		60	0.172	13.3	16.9	6.7×10^{-3}	

Table S5 Competition for Cu(I) between Bca and *apo*-proteins nA-PcoC, C42S-Rd and Atx1 in Mops buffer (50 mM, pH 7) and 100 mM NaCl.

$[Cu]_{Total}$ (μM)	$[Bca]_{total}$ (μM)	*Apo*-protein	$[P]_{total}$ (μM)	A_{562}	$[Cu(Bca)_2]^{3-}$ (μM)	$[Cu^I\text{-}P]$ (μM)	K_{ex}	Average K_{ex}
30.4	100-3000	none	0	0.240	30.4			
15.0	45	PcoC	10	0.074	9.5	5.5	8.8×10^{-5}	6.6×10^{-5}
			25	0.059	7.6	7.4	5.0×10^{-5}	
			50	0.044	5.6	9.4	4.6×10^{-5}	
			100	0.025	3.2	11.8	6.2×10^{-5}	
			150	0.015	1.9	13.1	8.4×10^{-5}	
31.1	2500	C42S-Rd	20	0.119	15.0	16.1	1.7	2.0
			30	0.067	8.5	22.6	2.2	
			40	0.041	5.2	25.9	2.2	
30.6	3000	Atx1	20	0.109	13.8	16.8	3.4	4.5
			30	0.052	6.6	24.0	5.5	

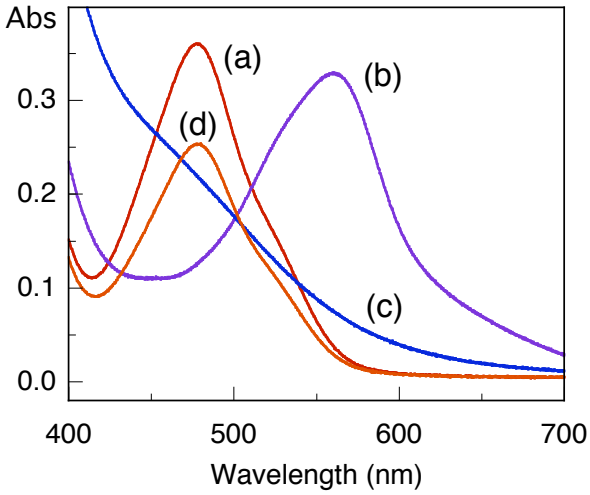

Figure S1. Solution spectra in buffer A:

(a) a solution of Cu^{2+} (40 μM), $GtsmH_2$ (50 μM) and Bca (200 μM);

(b) after addition of sodium dithionite (1 mM) into solution (a);

(c) after addition of sodium dithionite (1 mM) into solution (a) *in the absence of* Bca;

(d) after addition of Edta (100 μM) into solution (b), followed by bubbling air into the solution.

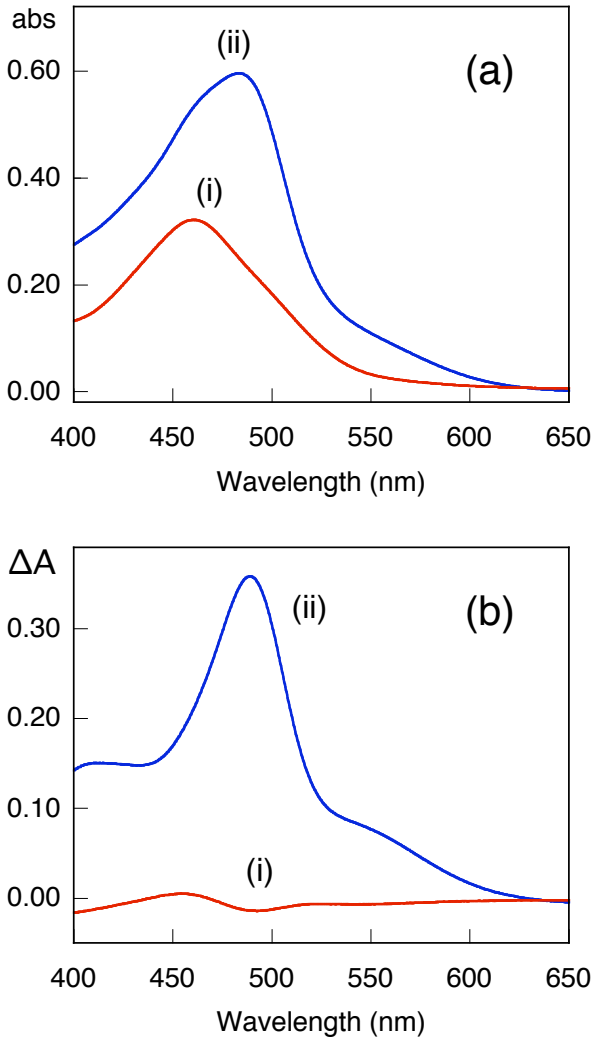

Figure S2. Solution spectra in buffer A:

(a) a mixture of Cu^{2+} (40 μM), $AtsmH_2$ (50 μM) and Bcs (1 mM) (i) and after reduction of the mixture with sodium dithionite (1 mM; 10 min) (ii);

(b) spectral difference recorded at 2 h after addition of sodium ascorbate (1 mM) or glutathione (1 mM) into solution a(i) (bottom red trace (i)) and at 10 min after addition of sodium dithionite (1 mM) (top blue trace (ii)).

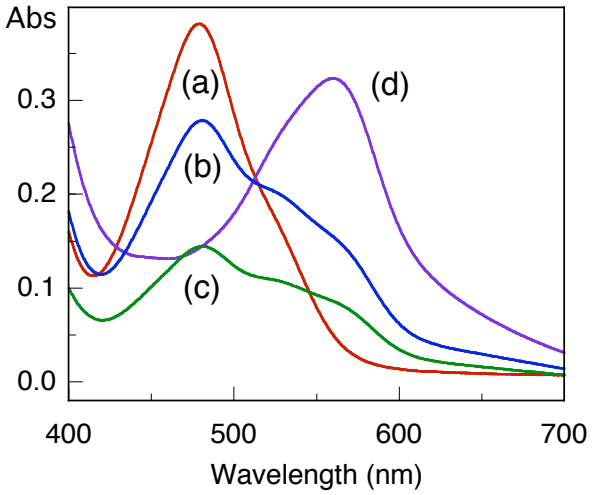

Figure S3. Solution spectra in buffer A:

(a) a mixture of Cu^{2+} (40 μM), $GtsmH_2$ (50 μM) and Bca (200 μM);

(b) after reduction of the solution (a) with sodium ascorbate (1 mM; 6 h);

(c) after reduction of the solution (a) with glutathione (1 mM; 6 h);

(d) after reduction of the solution (a) with sodium dithionite (1 mM; 10 min).

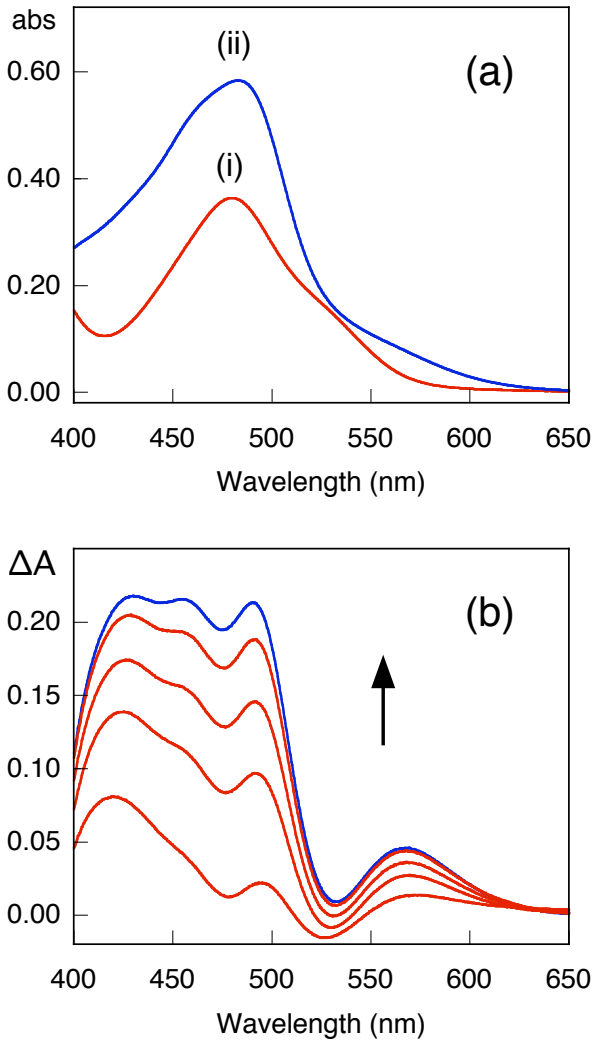

Figure S4. Solution spectra in buffer A:

(a) a mixture of Cu^{2+} (40 μM), $GtsmH_2$ (50 μM) and Bcs (1 mM) (i) and after reduction of the mixture with sodium dithionite (1 mM; 10 min) (ii);

(b) spectral difference recorded at 1, 2, 3, 5, 7 min (from bottom to top) after addition of GSH (1 mM) into solution a(i) (note: the top blue trace at 7 min superimposed the spectral difference between a(ii) and a(i)).

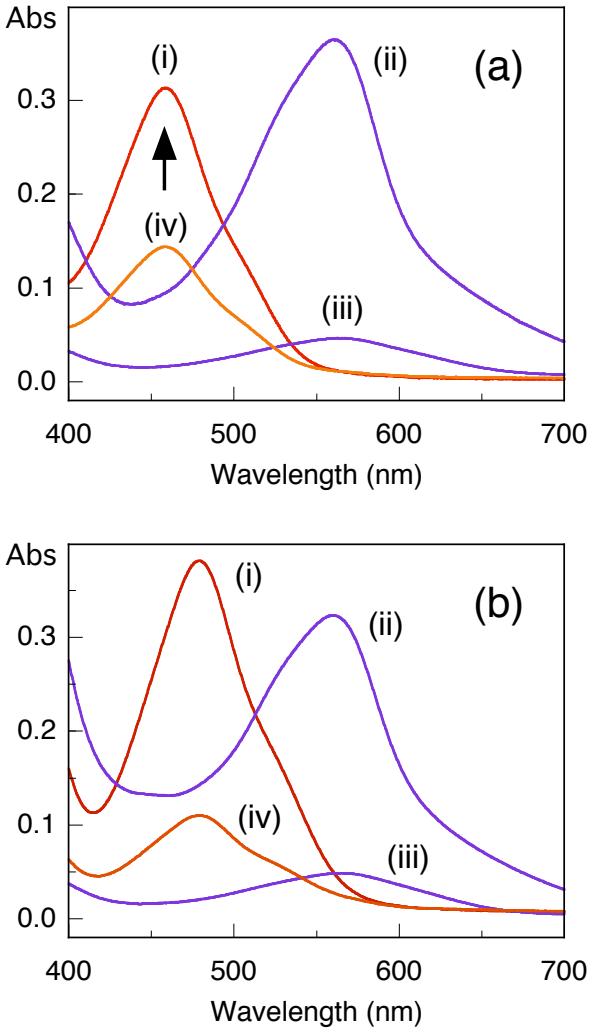

Figure S5. Solution spectra in buffer A containing LH_2 = $AtsmH_2$ (a) and $GtsmH_2$ (b):

(i) a solution of Cu^{2+} (40 μM), LH_2 (50 μM) and Bca (200 μM);

(ii) after addition of sodium dithionite (1 mM) into solution (i);

(iii) after addition of sodium dithionite (1 mM) and glutathione (1 mM) into solution (i);

(iv) (a) 10 min after bubbling air into solution a(iii) (note: the spectrum a(iv) was increasing in intensity with time and overlapped with spectrum a(i) in about 1 h); (b) 3h after bubbling air into solution b(iii) (note: the spectrum b(iv) remained little change in intensity with time).

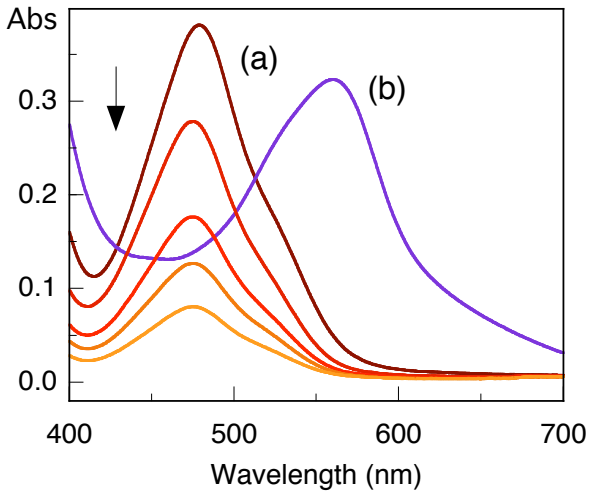

Figure S6. Solution spectra in KPi buffer (20 mM; pH 7; 100 mM NaCl; 10% DMSO):

(a) top, a mixture of $CuSO_4$ (40 μM), $GtsmH_2$ (50 μM) and BCA (200 μM) and from the second top,10min, 1h, 2h and 4h after addition of *apo*-Atx1 (100 μM) into the top solution;

(b) after addition of sodium dithionite (1 mM) into the top solution (a) *without apo*-Atx1.