Inorg. Chem. **2008**, *47*, 4338−4347

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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Received December 18, 2007

Bis(thiosemicarbazonato) complexes Cu^{II}(Btsc) have attracted interest as promising metallodrugs and, in particular, as copper radiopharmaceuticals. Prototypes Cu(Atsm) and Cu(Gtsm) are membrane-permeable, but their metabolisms in cells are distinctly different: copper that is delivered by Cu(Gtsm) is trapped nonselectively in all cells, whereas copper that is delivered by Cu(Atsm) is retained selectively in hypoxic cells but is "washed out" readily in normal cells. We have studied copper-transfer reactions of these two complexes under various conditions, aiming to model their cellular chemistry. In Me_2SO, both complexes exhibited reversible one-electron-reduction processes with Cu(Atsm) being more difficult to reduce than Cu(Gtsm) ($E_{1/2}' = -0.60$ and -0.44 V, respectively, vs AgCl/Ag). Upon introduction of an aqueous buffer into Me_2SO, the electrochemical reduction remained chemically reversible for Cu(Atsm) but became irreversible for Cu(Gtsm). However, the estimated difference in their reduction potentials did not change. Chromophoric ligand anions bicinchonate (Bca) and bathocuproine disulfonate (Bcs) were used as Cu^{I} indicators to trace the destinations of copper in the reactions and to mimic cellular Cu^{I}-binding components ("sinks"). While both $BtscH_2$ ligands have high affinities for Cu^{I} (K_D in the picomolar range), they cannot compete with Cu^{I} sinks such as the copper-binding proteins Atx1 and Ctr1c (or a mimic such as Bcs). In the presence of these proteins, reduction of Cu^{II}(Btsc) leads to irreversible transfer of copper to the protein ligands. Endogenous reductants ascorbate and glutathione can reduce Cu^{II}(Gtsm) in the presence of such protein ligands but cannot reduce Cu^{II}(Atsm). These properties establish a strong correlation between the contrasting cellular retention properties of these complexes and their different reduction potentials. The endogenous reductants in normal cells appear to be able to reduce Cu^{II}(Gtsm) but not Cu^{II}(Atsm), allowing the latter to be washed out. The more reducing environment of hypoxic cells leads to reduction of Cu^{II}(Atsm) and retention of its copper.

Introduction

^{64}Cu is a positron-emitting nuclide with a half-life of 12.7 h and the potential to be used as a tracer for positron emission tomography in noninvasive diagnostic imaging.[1,2] The isotope is best administered in the form of a stable coordination complex. The bis(thiosemicarbazone) (Btsc) complexes provide one promising delivery system because their complexes with Cu^{II} are stable, neutral, and relatively lipophilic.[3–6] Biodistribution of radiolabeled Btsc complexes is remarkably sensitive to the nature of the backbone substituents on the diimine backbone (Figure 1).[7,8] [Abbreviations: Asc, ascorbate anion; Atx1, copper chaperone from *Saccharomyces cerevisiae*; Atox1, human copper chaperone; Bca, bicinchonate anion; Bcs, bathocuproine disulfonate anion; bis(thiosemicarbazone), Btsc, bis(thiosemicarbazone); Ctr1c, protein expressed as residues 282–406 of the Ctr1 protein of *S. cerevisiae*; Cp, ceruloplasmin; Dtt, dithiothreitol; Edta,

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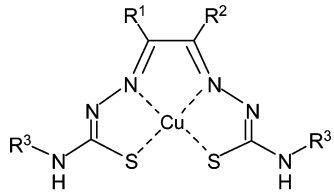
Published on Web 04/16/2008



$R^1 = R^2 = H; R^3 = CH_3$, Cu(Gtsm)
$R^1 = H; R^2 = R^3 = CH_3$, Cu(Ptsm)
$R^1 = R^2 = R^3 = CH_3$, Cu(Atsm)

Figure 1. Copper bis(thiosemicarbazonato) complexes and abbreviations.

N,*N*,*N*′,*N*′-ethylenediamine tetraacetic acid; GSH, glutathione; KPi, potassium phosphate buffer; SOD1, human superoxide dismutase.]

Low dioxygen concentration (hypoxia) has emerged as an important factor in tumor biology. Hypoxic tissue is associated with advanced solid tumors as the dioxygen consumption rate of the cancerous cells exceeds supply.[9] Correlations exist between hypoxia, tumor aggressiveness, angiogenesis, and response to treatment. Consequently, the development of hypoxia-selective imaging agents would help to guide therapeutic intervention. While Cu(Ptsm) has been tested as a blood perfusion tracer,[3] Cu(Atsm) has been investigated as a hypoxia-selective imaging agent.[10]

Copper is an essential element, but its cellular metabolism is controlled tightly to minimize potential toxic effects. A sophisticated array of specific membrane-bound pumps and chaperone proteins ensure nutrient balance (Figure 2).[11–13] These proteins exhibit high affinities for Cu^{I} (perhaps in the subfemtomolar range[11,14]) and are responsible for the safe delivery of essential copper to the right destinations. They acquire copper from membrane-bound copper pumps such as Ctr1 (Figure 2).

The trace concentrations of copper used in diagnostic imaging are designed to avoid a pharmacological response. Nonetheless, it is pertinent to consider the potential role of copper proteins in the metabolic retention of hypoxia-selective copper radiopharmaceuticals. Copper-binding proteins may be involved in sequestration of Cu^{I} made available by the reduction of Cu^{II}(Btsc) complexes. Metabolic studies in normal rat liver with a series of ^{64}Cu-labeled macrocyclic complexes revealed that the label appeared mainly in the copper proteins superoxide dismutase (SOD1), ceruloplasmin

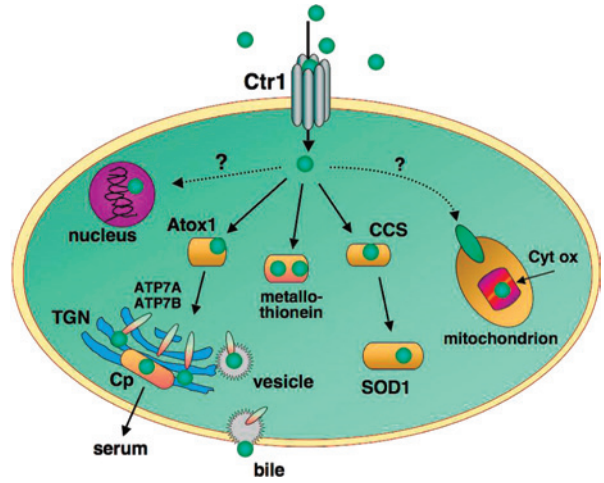


Figure 2. Schematic depiction of copper transport and distribution in a mammalian liver cell. Copper enters the cell via the high-affinity copper transporter Ctr1 and is transported to various locations. The pathway of copper delivery to the mitochondrion for incorporation into cytochrome *c* oxidase (Cyt ox) is not well-established and may involve many proteins including the Cox and Sco protein families. The chaperone CCS transfers copper to cytosolic SOD1, and Atox1 delivers copper to the *trans*-Golgi network (TGN) via the Cu-ATPase ATP7B (ATP7A in other cells), where copper is inserted into secreted copper-dependent enzymes such as ceruloplasmin (Cp). Under copper stress, ATP7B translocates via membrane vesicles to the cell membrane to expel excess copper.

Cp, and metallothionein (Figure 2).[15] In human cells, the copper chaperones CCS and Atox1 are respectively involved in trafficking of copper to SOD1 and the *trans*-Golgi network (TGN; where, in liver cells, Cp is generated). Metallothioneins are a class of cytosolic cysteine-rich small proteins with high affinities for soft metal ions such as Cu^{I} (Figure 2). In addition, the cytosolic cysteinyl tripeptide glutathione (GSH) may also help to scavenge labile Cu ions. These copper-binding peptides and proteins have little affinity for Cu^{II}, but their thiol functions may also act as effective reductants. Other possible bioreductants include ascorbate (Asc), nicotinamide adenine dinucleotides, flavins, and thioredoxins.[16]

Cu^{II}(Btsc) complexes are neutral and capable of crossing cell membranes. In the case of Cu(Gtsm) (Figure 1), the complex enters cells and the copper is trapped regardless of the oxygen concentration. In contrast, Cu(Atsm) enters cells, but the copper is retained only in hypoxic cells. Because Cu(Atsm) is harder to reduce than Cu(Gtsm), retention of Cu(Atsm) has been attributed to a reductive trapping mechanism involving the Cu^{II}/Cu^{I} couple.[7,17] Alternatively, different affinities of the ligands for Cu^{I} have been proposed as being influential in determining the contrasting behavior.[18,19] Redox enzymes, NADH-cytochrome b5 reductase,

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Table 1. Compositions of Buffer and Solutions Used

	composition
buffer A	KPi buffer (20 mM, pH 7; 100 mM NaCl) containing Me_2SO (30% v/v)
solution A	Cu^{2+} (40 μM), $AtsmH_2$ (50 μM) and Bca (200 μM) in buffer A
solution B	Cu^{2+} (40 μM), $GtsmH_2$ (50 μM) and Bca (200 μM) in buffer A
solution C	Cu^{2+} (40 μM), $GtsmH_2$ (50 μM) and Bcs (1000 μM) in buffer A
solution D	Cu^{2+} (40 μM), $AtsmH_2$ (50 μM) and Bcs (1000 μM) in buffer A

and NADPH-cytochrome P450 reductase have been implicated in the reductive retention of Cu^{II}(Atsm).[10,20]

We report a study in aqueous media of the reactions of the prototype complexes Cu^{II}(Btsc) (Btsc = Atsm or Gtsm; Figure 1) with known cellular reductants Asc and GSH and with the yeast copper-transport proteins Atx1 and Ctr1c under both oxidizing and reducing conditions. Chromophoric ligand anions bicinchonate (Bca) and bathocuproine disulfonate (Bcs) were used as Cu^{I} indicators to trace the destinations of copper in the reactions and to mimic cellular Cu^{I}-binding components ("sinks"). The study provides direct support for the reductive trapping mechanisms discussed above for copper uptake mediated by Cu^{II}(Btsc) complexes and for the hypoxia selectivity exhibited by Cu(Atsm).

Experimental Section

General Procedures. Btsc ligands were prepared according to literature procedures.[21,22] The Cu^{I}-binding proteins, Atx1 and Ctr1c (the C-terminal domain of the membrane copper transporter Ctr1), from *Saccharomyces cerevisiae* were expressed and purified as described previously.[14] They were isolated as the apo forms, and their cysteine residues were reduced by incubation overnight with dithithreitol (Dtt; 100 mM). The reductant was removed in an anaerobic glovebox ($[O_2]$ < 10 ppm) via an Econo-Pac 10DG desalting column (Bio-Rad). Other chemicals were purchased from the Sigma-Aldrich Chemical Co. Sodium ascorbate and dithionite solutions were freshly prepared daily in the glovebox. All reactions were carried out in buffer A (KPi, 20 mM, pH 7; NaCl, 100 mM; Me_2SO, 30% v/v; Table 1). Me_2SO was included to ensure solubility of both the Btsc ligands and the complexes. To minimize the possibility of precipitation, stock solutions of ligands were prepared in pure Me_2SO and the other components as aqueous solutions. For reactions, the stocks were mixed proportionally to give the desired reaction composition. All reactions were carried out in deoxygenated buffers in the glovebox, except where indicated. Reaction solutions were transferred in septum-sealed quartz cuvettes for spectroscopic characterization.

Concentration Assays. Stock solutions of $[Cu^{I}(MeCN)_4]ClO_4$, $CuSO_4$, Edta, Bca, and Bcs were standardized according to protocols described previously.[23] Concentrations of ligands $BtscH_2$ were calibrated by titration with a $CuSO_4$ standard that reacted with either ligand quantitatively to produce a 1:1 complex with a characteristic visible spectrum.[18] The concentrations of Atx1 and Ctr1c proteins were estimated from ϵ_{280} values determined previously.[14]

Characterization of Copper-Containing Species and Reactions. Each species was prepared separately by titration of $[Cu^{I}(MeCN)_4]ClO_4$ or $CuSO_4$ into the respective ligand or apoprotein solution and characterized spectroscopically.

To follow copper distribution and transfer reactions involving Btsc and other copper ligands under both oxidizing and reducing conditions, Cu^{2+} was titrated into a solution containing a specific ligand and other competing ligands (including apoproteins) under anaerobic conditions, followed by the addition of reductants. Oxidation of reduced solutions was effected by gently bubbling air through the solution. The distribution of copper at various reaction stages was monitored by spectroscopy and/or by separation of the protein components from other components of low molar mass via a protein concentrator (Centricon). The copper content of both the eluate and retentate fractions was determined by graphite cuvette atomic absorption spectroscopy (GC-AAS).

Estimation of the Binding Affinities of Ligands $BtscH_2$ for Cu^{I}. A previous protocol was employed.[23] Briefly, a series of solutions containing $[Cu^{I}(Bca)_2]^{3-}$ (λ_{max} = 562 nm; ϵ = 7900 M^{-1} cm^{-1}) were prepared by mixing $CuSO_4$ and Na_2Bca in a molar ratio of 1:2.5 in the presence of sodium dithionite (final concentration, 1 mM) followed by the quantitative addition of the ligand. Each solution was diluted to the same total concentration of Cu^{I} (40 μM) and Bca (100 μM) with varying total concentrations of the ligand. Competition for Cu^{I} was estimated by a decrease of the absorbance at 562 nm relative to a control solution without the ligand.

The formation constant β_2 for $[Cu^{I}(Bca)_2]^{3-}$ was estimated by indirect competition for Cu^{I} between Bca and Bcs mediated separately by three apoproteins (Atx1[14], nA-PcoC,[23] and C42S-rubredoxin[24]) that bind Cu^{I} with very different affinities. Details are provided in the Supporting Information.

Physical Measurements and Electrochemistry. Absorption spectroscopy was performed on a Varian Cary 50 spectrophotometer in dual-beam mode with quartz cuvettes of 1 cm path length. Steady-state fluorescence spectra were obtained on a Varian Cary Eclipse spectrophotometer using rectangular quartz cuvettes of 1 cm path length. The excitation wavelength was 290 nm with a bandpass of 10 nm for both excitation and emission.

Electrochemical experiments were carried out in Me_2SO with 0.1 M $Bu^n_4NBF_4$ as the electrolyte and in mixed solutions of Me_2SO (0.1 M $Bu^n_4NBF_4$) and an aqueous buffer (50 mM; pH 6–9; 100 mM NaCl) in a ratio of 3:2 (v/v). Limited solubility (micromolar range) in buffer A (Table 1) prevented such studies in that buffer. A standard three-electrode system was employed. The working electrode was a disk (diameter, 5 mm) of edge-cleaved pyrolytic graphite (PGE; Le Carbone-Lorraine) housed in a sheath of epoxy resin, and the counter electrode was a platinum wire. Observed potentials were measured relative to a KCl-saturated Ag/AgCl reference electrode that was calibrated with the methylviologen couple (–643 mV at 25 °C in a KPi buffer).[25]

Results and Discussion

Chemistry of Cu^{II}(Btsc) Complexes in Aqueous Buffer Solution. Previous investigations have employed anhydrous polar solvents due to the limited solubility in an aqueous

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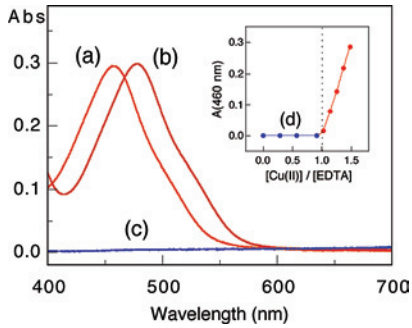

Figure 3. Solution spectra in buffer A: (a) Cu^{2+} (40 μM) and $AtsmH_2$ (50 μM); (b) Cu^{2+} (33 μM) and $GtsmH_2$ (50 μM); (c) after titration of Cu^{2+} (40 μM) into a *stirred* solution of $AtsmH_2$ or $GtsmH_2$ (50 μM) and Edta (100 μM); (d) change in A_{460} with a $[Cu^{II}]/[Edta]$ ratio during the course of titration (c) for ligand $AtsmH_2$.

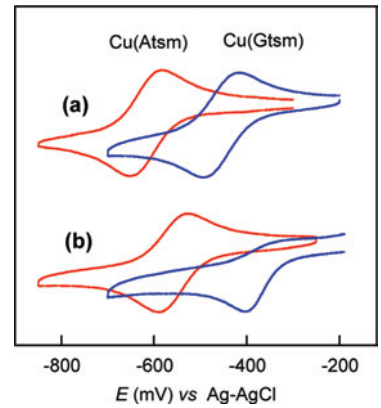

Figure 4. Cyclic voltammograms of Cu^{II}(Btsc) complexes (Btsc = Atsm or Gtsm; 1.0 mM) at a scan rate of 50 mV s^{-1}: (a) in Me_2SO (0.1 M $Bu^n_4NBF_4$); (b) in a mixed solution of Me_2SO (0.1 M $Bu^n_4NBF_4$) and an aqueous buffer (50 mM; pH 6–9; 100 mM NaCl) in a ratio of 3:2 (v/v). The buffers used were KPi (pH 6 and 7), Tris-Cl (pH 8), and Na-Ches (pH 9).

solution of each ligand and its Cu^{II} complex. To model cellular chemistry more adequately, a KPi buffer containing 30% (v/v) Me_2SO (buffer A; Table 1) was chosen. The complexes Cu^{II}(Btsc) dissolve in this medium in micromolar concentrations, as do many ionic solutes. Its dielectric constant ($\epsilon \sim 77$) is close to that of water.[26]

Titration of a Cu^{2+} solution into a solution of $AtsmH_2$ in buffer A (solution A; Table 1) produced the orange complex Cu^{II}(Atsm) quantitatively. It is characterized by an absorption maximum ($\lambda_{max} = 457$ nm; $\epsilon = 7200$ M^{-1} cm^{-1}; Figure 3a) blue-shifted by 19 nm from that observed in pure Me_2SO.[18] The equivalent reaction with $GtsmH_2$ produced Cu^{II}(Gtsm) with an absorption maximum ($\lambda_{max} = 478$ nm; $\epsilon = 8700$ M^{-1} cm^{-1}), which was again blue-shifted by 19 nm from that found in pure Me_2SO (Figure 3b).

These Cu^{II} complexes are stable and inert in buffer A. For example, the solution spectrum of Cu^{II}(Atsm) remained unchanged after overnight incubation with Edta (1000 equiv). However, titration of Cu^{2+} into a *stirred* solution containing both $AtsmH_2$ and Edta (molar ratio, 1:2) provided no detectable absorbance, even after incubation of the resultant solution overnight (Figure 3c). The latter observations are consistent with the formation of Cu^{II}(Edta) because only at molar ratios Cu^{II}/Edta > 1 does the characteristic spectrum of Cu^{II}(Atsm) appear (Figure 3d). It is apparent that Edta binds Cu^{2+} more rapidly than does $AtsmH_2$ and that Cu^{II} does not exchange between these two ligands.[27] $GtsmH_2$ provided equivalent results.

Consistent with a previous study,[28] Cu(Atsm) is more difficult to reduce than is Cu(Gtsm). Each exhibited a well-developed cyclic voltamogram in anhydrous Me_2SO (0.1 M Bu_4NBF_4) with respective midpoint reduction potentials $E_{1/2}'$ [$=(E_p^{red} + E_p^{ox})/2$] of –0.60 and –0.44 V (vs AgCl/Ag; Figure 4a and Table S1 in the Supporting Information). For scan rates in the range of 20−1000 mV s^{-1}, I_p^{ox}/I_p^{red} parameters were close to unity and ΔE_p was 65(5) mV for a scan rate of 50 mV s^{-1}. The difference from the theoretically predicted value of 56 mV at 20 °C for a reversible one-electron process can be attributed to a low level of uncompensated resistance.[29] The data are consistent with the following reversible processes:

$$Cu^{II}(Btsc) \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} [Cu^{I}(Btsc)]^- \qquad (1)$$

Upon introduction of a KPi buffer (50 mM; pH 7) into a Me_2SO solvent (2:3, v/v), the process remained chemically reversible for Cu^{II}(Atsm) and $E_{1/2}'$ showed a positive shift of $\sim$ 40 mV (Figure 4b and Table S1 in the Supporting Information). This shift in potential is most probably associated with perturbation of the reference electrode upon a change of the solvent. A similar positive shift was observed for the redox couple $[Fe(CN)_6]^{3-/4-}$ under the same conditions. On the other hand, the process became irreversible for Cu^{II}(Gtsm) under the same conditions (Figure 4b). Within experimental error, the $E_{1/2}'$ values did not vary with the pH of the buffer component in the range pH 6–9 (Table S1 in the Supporting Information). This behavior suggests that, under these conditions, the reduction is not coupled to protonation of the product anions $[Cu^{I}(Btsc)]^-$ within this pH range. A previous study in pure Me_2SO in the presence of proton donors led to similar conclusions.[18]

These experiments suggest that the anion $[Cu^{I}(Atsm)]^-$ is stable in this protonic medium on the experimental time scales (eq 1) but that $[Cu^{I}(Gtsm)]^-$ undergoes significant structural reorganization or a following chemical reaction (which may include dissociation) on the same time scale. An air-sensitive binuclear salt $[Cu^{I}_2(AtsmH_2)_2][PF_6]_2$ has been isolated by reaction of $[Cu^{I}(MeCN)_4][PF_6]$ with $AtsmH_2$ in MeCN.[30] The neutral $AtsmH_2$ ligands act as bidentate N−S bridges to the binuclear center. Its relevance to the present results is uncertain.

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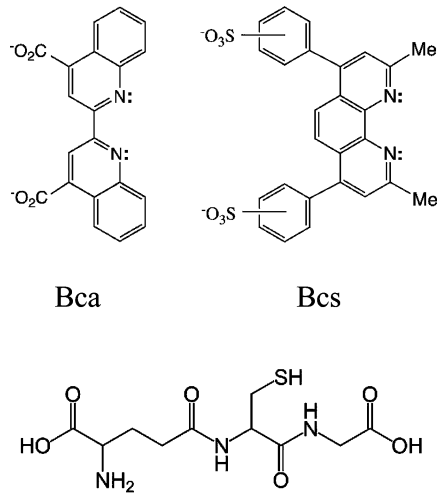


Figure 5. Ligands bicinchonate anion (Bca), bathocuproine disulfonate (Bcs), and glutathione (γ-Glu-Cys-Gly; GSH).

Reactivity of Cu^{I} Complexes. Given the redox properties discussed above, intracellular reduction of the Cu^{II}(Btsc) complexes may induce competition for Cu^{I} among various copper sinks. To identify possible processes, selected ligands and proteins were tested for their ability to extract Cu^{I} from Btsc ligands upon reduction of Cu^{II}(Btsc) complexes (Btsc = Atsm and Gtsm).

Dianionic ligand Bca (Figure 5) is an excellent Cu^{I} indicator and reacts with Cu^{+} specifically to form the complex anion $[Cu^{I}(Bca)_2]^{3-}$ whose absorption maximum (λ_{max} = 562 nm; ϵ = 7900 M^{-1} cm^{-1}) is separated from those of Cu^{II}(Btsc).[31] This anion is stable in air and can be generated readily by the reaction of Cu^{2+} and Bca in the presence of a reductant such as Asc, NH_2OH, or dithionite. Its formation constant was determined to be $\beta_2 = 2(1) \times 10^{17}$ M^{-2} (see Table S3 in the Supporting Information).

Titration of Cu^{2+} into solutions containing $BtscH_2$ and Bca in buffer A in molar ratios of 1:1.25:5 produced solutions A and B (Table 1), whose visible spectra were identical with those of Cu^{II}(Btsc) (Figures 3a, 6a, and S1a in the Supporting Information). Subsequent addition of the strong reductant dithionite[32] induced an immediate color change from orange to purple (Figures 6b and S1b in the Supporting Information). The product spectrum is consistent with >90% reductive transfer of copper from Cu^{II}(Btsc) to $[Cu^{I}(Bca)_2]^{3-}$. Apparently, both reduced species $[Cu^{I}(Btsc)]^{-}$ (eq 1) are labile and the affinity of $BtscH_2$ for Cu^{I} was too weak to retain Cu^{I} in the presence of excess Bca. The addition of dithionite to Cu^{II}(Btsc) in the absence of Bca caused immediate bleaching (Figures 6c and S1c in the Supporting Information), again signaling reduction of the bound Cu^{II} centers.

Excess dithionite was destroyed by the bubbling of air into each of the reduced solutions. Rapid and quantitative recovery of Cu^{II}(Btsc) was observed in both cases, i.e., Figure 6b,c converted to Figure 6a and Figure S1b,c to Figure S1a in the Supporting Information. It is apparent that a change in the oxidation state induced shuttling of copper between the two ligands:

$$Cu^{II}(Btsc) + 2Bca^{2-} + 2H^{+} \underset{+O_2}{\overset{+e^-}{\rightleftharpoons}} [Cu^{I}(Bca)_2]^{3-} + BtscH_2 \quad (2)$$

Forward reaction 2 is a model of cellular copper uptake where ligand Bca is mimicking a Cu^{I} sink. $[Cu^{I}(Bca)_2]^{3-}$ is stable in air, but, interestingly, when reverse reaction 2 was carried out in the presence of Edta (2 equiv), 70–80% of the total copper was converted to Cu^{II}(Btsc) and 20–30% was trapped as Cu^{II}(Edta) (Figures 6d and S1d in the Supporting Information). In contrast, as demonstrated in Figure 3c,d, titration of Cu^{2+} into a *stirred* solution containing $BtscH_2$:Edta = 1:2 led to complete sequestration of Cu^{II} by Edta only. In addition, there was no detectable exchange of Cu^{II} between the ligands Edta and $BtscH_2$ for at least 24 h. Consequently, given that $[Cu^{I}(Bca)_2]^{3-}$ is itself stable in air, preferential capture of Cu^{II} by the ligands $BtscH_2$ from $[Cu^{I}(Bca)_2]^{3-}$ must occur via the formation of a transient species such as $[Cu^{I}(Btsc)]^{-}$ (eq 1) that formed via *direct* exchange of Cu^{I} between $BtscH_2$ and Bca (Scheme 1). The appearance of minor product Cu^{II}(Edta) would follow from less efficient oxidative extraction of Cu^{II} by Edta, in combination with O_2, from $[Cu^{I}(Bca)_2]^{3-}$ and/or $[Cu^{I}(Btsc)]^{-}$. Separate controls demonstrated that Edta is able to extract copper from $[Cu^{I}(Bca)_2]^{3-}$ in the presence of O_2 but *not* under reducing conditions (such as in the presence of dithionite; see Figure 7). Therefore, Cu^{I} exchange cannot occur between Edta and Bca.

The above observations indicate that both ligands $BtscH_2$ have a lower affinity for Cu^{I} than for Cu^{II} and that, in contrast

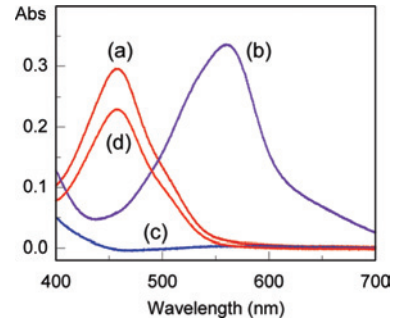
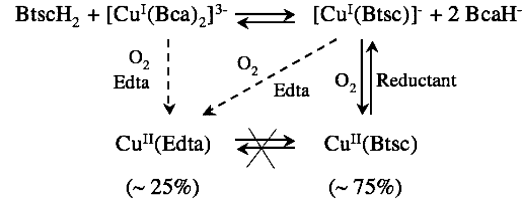


Figure 6. Solution spectra in buffer A: (a) solution A containing Cu^{2+} (40 μM), $AtsmH_2$ (50 μM), and Bca (200 μM); (b) after the addition of sodium dithionite (1 mM); (c) after the addition of sodium dithionite (1 mM) into solution (a) *in the absence of* Bca; (d) after the addition of Edta (100 μM) into solution (b), followed by the bubbling of air into the solution.

Scheme 1



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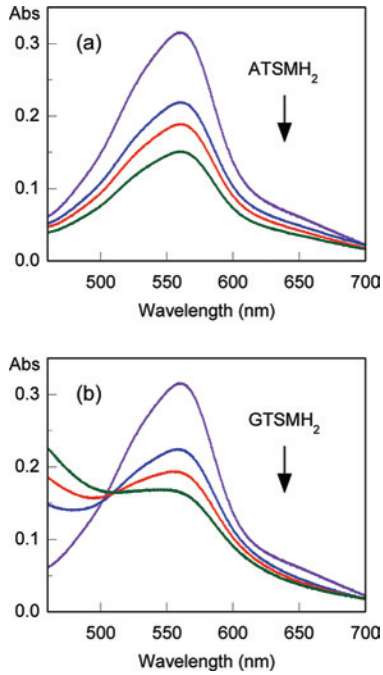

Figure 7. Change in the absorption intensity for a $[Cu^{I}(Bca)_2]^{3-}$ anion formed by mixing Cu^{2+} (40 μM), Bca (100 μM), and dithionite (1 mM) under *anaerobic* conditions in buffer A with increasing ligand concentrations: (a) $AtsmH_2$; (b) $GtsmH_2$. From top to bottom: total ligand concentration = 0, 50, 100, and 200 μM. The addition of Edta (200 μM) into the first top solution has no effect on the absorption intensities.

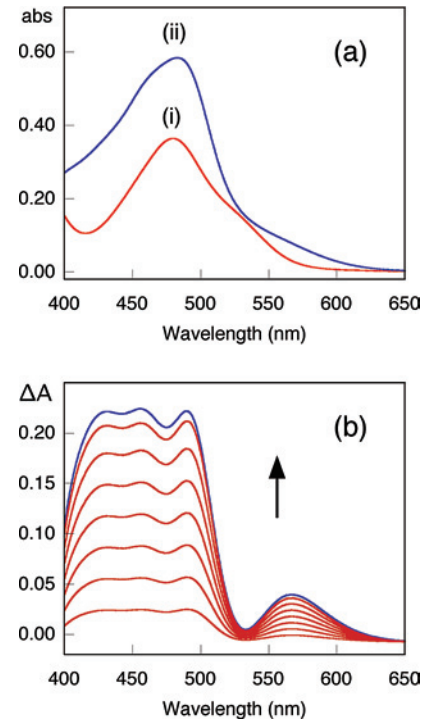

Figure 8. Solution spectra of a mixture containing Cu^{2+} (40 μM), $GtsmH_2$ (50 μM), and Bcs (1 mM) in buffer A upon the addition of reductants: (a) before (i) and after (ii) the addition of sodium dithionite (1 mM; 10 min); (b) difference spectra recorded every 8 min after the addition of sodium ascorbate (1 mM) into solution a(i) [note: the top blue trace is the spectral difference between a(ii) and a(i)].

to Edta, they are able to bind and exchange Cu^{I} directly with other Cu^{I}-binding ligands. However, as demonstrated in Figure 4, the reduction in protonic solvents is chemically reversible for Cu^{II}(Atsm) but not for Cu^{II}(Gtsm). One implication is that $GtsmH_2$ may have a lower affinity for Cu^{I} and reduction may lead to dissociation. It has been suggested that the differences in hypoxia selectivity exhibited by Cu^{II}(Atsm) and Cu^{II}(Gtsm) may be attributed partially to their difference in Cu^{I} affinity:[18,19] $AtsmH_2$ was assumed to retain Cu^{I} more effectively than $GtsmH_2$ and thus, upon oxygen exposure, was able to trap Cu^{I} more efficiently as membrane-permeable Cu^{II}(Atsm).

Affinities of Ligands $BtscH_2$ for Cu^{I}. To test the above speculations, experiments were designed to determine and compare the binding affinities of ligands $BtscH_2$ and Edta for Cu^{I} in the presence of the minimum concentration of tracer Bca (1:2.5) necessary to maintain $[Cu^{I}(Bca)_2]^{3-}$ as the sole Cu^{I}-Bca complex present in the control solution.

The addition of $BtscH_2$ (0–200 μM) to a $[Cu^{I}(Bca)_2]^{3-}$ solution (Cu^{2+}, 40 μM; Bca, 100 μM; dithionite, 1 mM) led to a proportional drop of the $[Cu^{I}(Bca)_2]^{3-}$ absorbance maximum at 562 nm, and the magnitude of that drop is similar for each ligand at the same concentration (Figure 7a,b). Consequently, the ligands $AtsmH_2$ and $GtsmH_2$ possess comparable affinities for Cu^{I}. The addition of Edta (200 μM) into the same $[Cu^{I}(Bca)_2]^{3-}$ solution caused no change in the absorbance, confirming that Edta has little affinity for Cu^{I}.

Under these conditions, it appears that the irreversible reduction of Cu^{II}(Gtsm) in protonic solvents (Figure 4) must be attributed to a structural change upon reduction rather than to the release of Cu^{+} into the solution (dissociation). A detailed analysis of the ligand competition data with experiments carried out in tandem with the same reagents (see Table S2 in the Supporting Information) revealed that $K_D \sim 10^{-13}$ M for both ligands and that $GtsmH_2$ has the higher affinity. However, these minor differences in Cu^{I} binding affinities are unlikely to be the cause of the striking difference in hypoxia selectivity.

The favored directions of reactions such as those in Scheme 1 will depend upon the relative Cu^{I} affinities of the competing ligands and also upon the balance between reduction and oxidation. Bathocuproine disulfonate (Bcs; Figure 5) has a higher affinity for Cu^{I} ($\beta_2 = 10^{19.8}$) than does Bca and, at 1 mM concentration, its affinity for Cu^{I} is comparable with those of protein thiols at subfemtomolar concentrations.[14] It reacts with Cu^{+} quantitatively to form the stable 1:2 orange complex anion $[Cu^{I}(Bcs)_2]^{3-}$ (λ_{max} = 483 nm; ϵ = 13 000 M^{-1} cm^{-1}). Its absorption maximum overlaps with those of Cu^{II}(Btsc), but its molar absorptivity is 4–5000 units higher (Figure 8a) and so reductive transfer of copper from Cu^{II}(Btsc) to $[Cu^{I}(Bcs)_2]^{3-}$ can be monitored sensitively by difference spectroscopy.

The addition of dithionite (1 mM) into solutions C and D (containing ligand $BtscH_2$ and Bcs (1 mM) as Cu^{I} indicators; Table 1) led to rapid, quantitative, and irreversible trapping of Cu^{I} as $[Cu^{I}(Bcs)_2]^{3-}$ (Figures 8a and S2 in the Supporting Information and eq 3):

$$Cu^{II}(Btsc) + 2Bcs^{2-} + 2H^{+} \xrightarrow{+e^{-}} [Cu^{I}(Bcs)_2]^{3-} + BtscH_2 \quad (3)$$

Exposure of the product solution to air did not induce the oxidative transfer of copper in the backward direction (cf. Scheme 1). Apparently, in the presence of excess free ligand Bcs, the oxidation reaction is not favored thermodynamically.

The difference in the reduction potentials between Cu^{II}(Atsm) and Cu^{II}(Gtsm) may be the major determinant of the favored direction of intracellular reactions related to Scheme 1 (i.e., ones in which endogenous ligands compete with $BtscH_2$ for Cu^{I}). However, the strong reductant dithionite employed so far reduces both Cu^{II}(Bstc) complexes and so cannot distinguish differences in their behavior. Consequently, known biological reductants, in combination with various Cu^{I} sinks, were employed for a more general investigation of the reactions in Scheme 1.

Reactions with Ascorbate. This reductant is considered to be the most important and effective antioxidant in human blood plasma (20–100 μM).[33,34] Intracellular concentrations can be as much as 100-fold higher because of active cellular transportation.[35]

Replacement of reductant dithionite in Scheme 1 by Asc (1 mM) in solution A (Table 1) caused no change in the spectrum of Cu^{II}(Atsm). Asc was unable to reduce Cu^{II}(Atsm) with Bca (200 μM) as the Cu^{I} sink. The equivalent addition of Asc to solution B (Table 1) induced slow reduction of Cu^{II}(Gtsm) ($t_{1/2} > 10$ h), with Cu^{I} being trapped as $[Cu^{I}(Bca)_2]^{3-}$ (Figure S3b in the Supporting Information). However, the reaction is too slow to be biologically significant. Notably, the affinities of most intracellular protein thiols for Cu^{I} are higher than that of Bca at 200 μM (see Table S5 in the Supporting Information), and in the presence of stronger Cu^{I} scavengers, Asc may be able to promote more efficient reduction of Cu^{II}(Gtsm) under cellular conditions.

The addition of Asc (1 mM) into solution C (Table 1) induced a quantitative spectral change from Cu^{II}(Gtsm) to $[Cu^{I}(Bcs)_2]^{3-}$ in about 1 h (Figure 8b). The experiment demonstrated that Asc is a viable reductant for Cu^{II}(Gtsm) in the presence of the stronger Cu^{I} sink Bcs (but not in the presence of Bca). The affinity of Bcs for Cu^{I} at 1 mM is comparable with protein thiols at subfemtomolar concentration.[14] The results demonstrate the importance of the relative Cu^{I} affinities of the competing ligands in any proposed reductively assisted transfer of copper. They suggest that, under intracellular conditions, Asc might induce reductive transfer of copper from Cu^{II}(Gtsm) to Cu^{I} sinks.

In contrast, however, Cu^{II}(Atsm) remained unreactive under the same conditions (Figure S2b in the Supporting Information), consistent with its lower reduction potential (Figure 4 and Table S1 in the Supporting Information).

Reactions with Glutathione. The cysteinyl tripeptide glutathione (GSH; Figure 5) is the low molar mass thiol in the cytosol of cells (0.5–10 mM). It participates in many cellular reactions and, in particular, functions as a redox buffer, scavenging free radicals and maintaining protein thiols in their reduced form.[36] It may also function as a metal buffer by acting as a ligand for soft metal ions such as Cu^{I}.

Experiments similar to those as depicted in Scheme 1 (without Edta) were carried out in solutions A−D (Table 1) with GSH as the reductant. Again, Cu^{II}(Atsm) was not reduced in any case, but in the presence of the higher affinity Cu^{I} ligand Bcs, Cu^{II}(Gtsm) was reduced efficiently ($t_{1/2} \sim 3$ min; Figure S4 in the Supporting Information). However, with its single thiol function, ligand GSH could not compete effectively with Bcs for Cu^{I} under the conditions and the copper was trapped exclusively as $[Cu^{I}(Bcs)_2]^{3-}$. Consequently, the literature value of $\beta_2 = 10^{39}$ M^{-1} for the formation of $[Cu^{I}(GS)_2]^{-}$ appears to significantly overestimate the affinity of GSH for Cu^{I}.[37] Separate experiments using dithionite as the reductant indicated that GSH could compete with Bca for Cu^{I} and that O_2 can readily "wash out" Cu^{I} trapped in the form of $[Cu^{I}(GS)_2]^{-}$ as Cu^{II}(Atsm) but not as Cu^{II}(Gtsm) (Figure S5 in the Supporting Information).

Reactions with Cu^{I}-Binding Proteins Atx1 and Ctr1c. The chaperone Atx1 from the yeast *Saccharomyces cerevisiae* delivers Cu^{I} specifically to the transport ATPase Ccc2 for entry into the TGN and eventual incorporation into the multicopper oxidase Fet3.[13] Both Atx1 and Ccc2 proteins contain the conserved metal-binding motif MT/HCXXC that has been demonstrated to bind Cu^{I} with subfemtomolar affinity.[14] Human homologues of Atx1, Ccc2, and Fet3 are Atox1, Menkes/Wilson proteins, and Cp, respectively (Figure 2).[38]

Copper is transported into the yeast cell via the plasma-membrane protein Ctr1 (cf. Figure 2).[39] Yeast Ctr1 features a cytosolic C-terminal domain (Ctr1c) composed of about 125 amino acid residues with six Cys residues. Similar to metallothioneins (Figure 2), apo-Ctr1c is largely unstructured but exhibits a high affinity for Cu^{I} and binds four Cu^{I} ions cooperatively as a $[Cu^{I}_4(\mu\text{-S-Cys})_6]^{2-}$ cluster with average $K_D \sim 10^{-19}$ M (Figure 9a).[14] A probe for the formation of this cluster in solution is reported here: titration of Cu^{+} into apo-Ctr1c induces luminescence at 610 nm with excitation at 290 nm (Figure 9b). This is consistent with the cluster $[Cu^{I}_4(\mu\text{-S-Cys})_6]^{2-}$ being protected from the solvent.[40]

Both Atx1 and Ctr1c were reacted with Cu^{II}(Btsc) under conditions similar to those described in Scheme 1. The addition of Atx1 (100 μM) or Ctr1c (20 μM) to solution A (Table 1) caused no change to the absorption spectrum of

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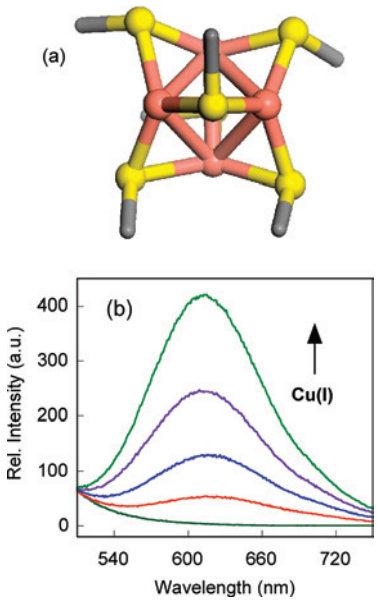

Figure 9. (a) Ball-and-stick presentation of the $[Cu^{I}_4(\mu\text{-S-Cys})_6]^{2-}$ cluster in Cu_4Ctr1c. (b) Emission spectra for titration of apo-Ctr1c [4 μM; 20 mM KPi (pH 7)] with $[Cu^{I}(MeCN)_4]^{+}$ (from bottom to top: 0, 4, 8, 12, and 16 μM).

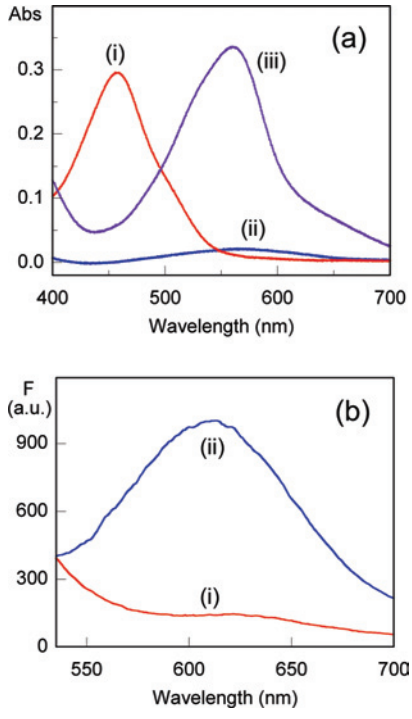

Figure 10. (a) Spectra in solution A [$CuSO_4$ (40 μM), $AtsmH_2$ (50 μM), and Bca (200 μM)] in the presence of apoproteins apo-Ctr1c (20 μM) or apo-Atx1 (100 μM): (i) initial spectrum (remained unchanged for at least 2 h); (ii) after the addition of sodium dithionite (1 mM); (iii) after the addition of sodium dithionite (1 mM) in the absence of proteins. (b) Emission spectra in buffer A upon excitation at 290 nm: (i) solution a(i) with apo-Ctr1c (20 μM); (ii) solution a(ii) with apo-Ctr1c (20 μM).

Cu^{II}(Atsm) for at least 2 h [Figure 10a(i)]. Neither protein was able to extract Cu^{II} directly from Cu^{II}(Atsm), and the relatively low protein concentrations employed in these

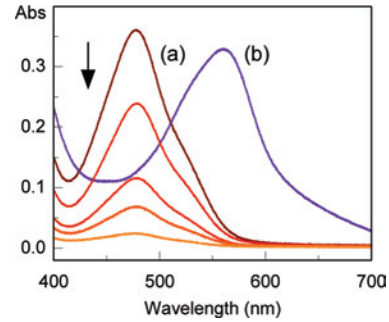

Figure 11. Solution spectra in buffer A [$CuSO_4$ (40 μM), $GtsmH_2$ (50 μM), and Bca (200 μM)]: (a) initial spectrum, followed by those taken 10, 30, 60, and120 min after the addition of apo-Ctr1c (~20 μM); (b) after the addition of sodium dithionite (1 mM) to the initial spectrum (in the absence of protein).

experiments were not sufficient to promote reduction of Cu^{II}(Atsm) by the protein thiols.

However, the addition of dithionite (1 mM) led to an immediate spectral change from Figure 10a(i) to Figure 10a(ii). Weak absorbance at 562 nm characteristic of $[Cu^{I}(Bca)_2]^{3-}$ was apparent in the spectrum in Figure 10a(ii), but the intensity was less than 10% of a control solution lacking proteins (Figure 10a(iii)). Therefore, Cu^{I} as putative $[Cu^{I}(Atsm)]^{-}$ was trapped predominately by Atx1 or Ctr1c. For example

$$4Cu^{II}(Atsm) + Ctr1c + 8H^{+} \underset{+O_2,\ slow}{\overset{+dithionite}{\rightleftharpoons}} Cu^{I}_4\text{-}Ctr1c + 4AtsmH_2 \quad (4)$$

This interpretation was confirmed by observation of luminescence at 610 nm characteristic of the $[Cu^{I}_4(\mu\text{-S-Cys})_6]^{2-}$ cluster in Ctr1c [Figure 10b(ii)], which was not detected in the same solution before dithionite reduction [Figure 10b(i)]. The conclusions were further substantiated by separation of the protein fractions from low molar mass fractions via a YM3 Centricon filter followed by estimation of the copper content in each fraction by GC-AAS. More than 95% of the copper was retained in the protein fractions.

Interestingly, the above separation of the protein fraction via a Centricon filter was carried out in air. The copper bound in Atx1 and Ctr1c was not transferred to the $AtsmH_2$ ligand present in the solution during the time scale of the separation (~2 h); i.e., the oxidative back reaction (4) was very slow. The cysteinyl ligands did oxidize upon extended exposure to air (overnight), leading to the slow release of copper, which was trapped subsequently as Cu^{II}(Atsm).

The above experiments required reduction of Cu^{II}(Atsm) with dithionite before transfer of copper to the proteins (eq 4 and Figure 10). In contrast, however, the addition of excess apo-Ctr1c to orange solution B containing Cu^{II}(Gtsm) (Table 1) led to a loss of that complex ($t_{1/2}$ ~ 15 min) with no need for an extra external reductant (eq 5 and Figure 11a):

$$4Cu^{II}(Gtsm) + Ctrlc + 8H^{+} \underset{+O_2,\ slow}{\rightleftharpoons} Cu^{I}_4\text{-}Ctr1c + 4GtsmH_2 \quad (5)$$

$[Cu^{I}(Bca)_2]^{3-}$ was not detected, but cluster $[Cu^{I}_4(\mu\text{-S-Cys})_6]^{2-}$ was detected by luminescence [Figure 10b(ii)]. GC-AAS analysis of the separated protein and nonprotein

fractions confirmed the copper destination. A combination of the reducing power and the high affinity for Cu^{I} of apo-Ctr1c[14] led to *complete* reductive transfer of copper from Cu^{II}(Gtsm) to Cu^{I}_{4}Ctr1c (eq 5 and Scheme 1).

The addition of apo-Atx1 (100 μM) into solution B also bleached the orange color of Cu^{II}(Gtsm), but the process was slower ($t_{1/2} \sim 1$ h; Figure S6 in the Supporting Information). Again, the differences in behavior of Cu^{II}(Atsm) and Cu^{II}(Gtsm) can be attributed to the latter being more easily reduced than the former.

Insights into Copper Uptake and Hypoxia Selectivity. Neutral Cu^{II}(Btsc) complexes are kinetically inert and thermodynamically stable and do not exchange Cu^{II} readily with other ligands. An oversimplified model of cellular uptake of copper delivered by these complexes is presented in Figure 12. These Cu^{II}(Btsc) complexes can enter cells readily but may be "washed out" because of their membrane permeability. The balance between cellular retention and wash-out of copper delivered by these complexes is influenced by their reduction potentials, which are controlled by the nature of the ligand substituents, especially R^1 and R^2 on the ligand backbone (Figure 12). Copper delivered by Cu(Gtsm) is trapped nonselectively in all cells. On the other hand, copper that is delivered by Cu(Atsm), which has a more negative reduction potential, is retained selectively in hypoxic cells but is "washed out" readily in normal cells.

This work presents the first significant study of the chemistry of these complexes in aqueous media. While both $BtscH_2$ ligands have high affinities for Cu^{I} (K_D in the picomolar range), they cannot compete with intracellular Cu^{I} sinks such as the proteins Atx1 and Ctr1c. In the presence of these protein ligands (or a mimic such as Bcs), reduction of Cu^{II}(Btsc) leads to an irreversible loss of copper from the Btsc ligand. The endogenous species Asc and GSH are ineffective reductants of Cu^{II}(Btsc) complexes. However, in the presence of the protein ligands, both reductants can induce rapid reductive transfer of copper from Cu^{II}(Gtsm) to Cu^{I}-binding proteins but *not* from Cu^{II}(Atsm). These properties establish a strong correlation between the contrasting cellular retention properties of these complexes and their different reduction potentials. The endogenous reductants in normal cells are able to reduce Cu^{II}(Gtsm) but not Cu^{II}(Atsm), allowing the latter to be washed out. The more reducing environment of hypoxic cells leads to reduction of Cu^{II}(Atsm) and retention of copper. The transfer of copper from Cu(Gtsm) to copper proteins is significant to our recent work, which demonstrated that this complex and its analogues can be used to deliver bioavailable copper to cell lines that are of relevance to Alzheimer's disease.[41]

Interestingly, a large proportion of metabolized ^{64}Cu imported via labeled macrocyclic complexes into rat liver cells was found to be bound to superoxide dismutase

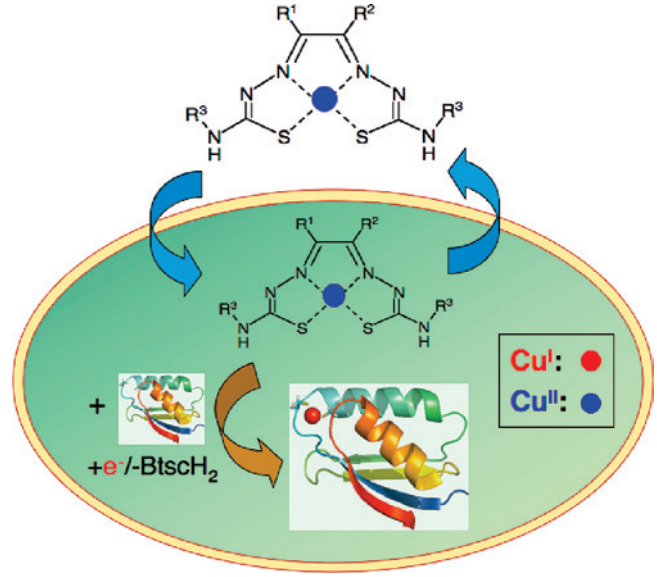

Figure 12. Oversimplified representation of the cellular uptake of copper delivered by Cu^{II}(Btsc) complexes. A balance between the uptake and wash-out of copper delivered by Cu^{II}(Btsc) is influenced strongly by the ligand backbone substituents R^1 and R^2. Upon reduction of Cu^{II}(Btsc), Cu^{I} in the complex is transferred to Cu^{I}-binding proteins such as the copper chaperone Atx1.

(SOD1; Figure 2).[15] SOD1 is an abundant (~10 μM) cytosolic Cu−Zn enzyme. It receives copper from its chaperone CCS under copper-limiting conditions (Figure 2) but also can accept "free" copper directly at elevated copper levels.[11] Chaperone CCS possesses three distinct cysteine-rich protein domains[42] that may facilitate copper uptake in SOD1 via Cu^{II} reduction (particularly in the presence of cytosolic Asc and/or GSH) coupled to Cu^{I} translocation.

Petering has reported that titration of Ehrlich cells with the Cu^{II}(Btsc) complex 3-ethoxy-2-oxobutyraldehydebis(thiosemicarbazonato)copper(II) (Figure 1) led to reductive transfer of copper to metallthionein (Figure 2).[43] When taken with the present work, these previous studies suggest a model in which cellular reduction of Cu^{II} complexes leads to activation and an irreversible loss of copper. It is recommended that the Cu^{II}/Cu^{I} reduction potentials of prospective copper radiopharmaceuticals be considered carefully, in addition to acid–base stabilities and kinetic inertness. For example, it has been reported that several Cu^{II} complexes of cross-bridged tetraazamacrocyles exhibit comparable kinetic inertness in acid but that those with more negative reduction potentials display superior in vivo stability.[44] In addition, a series of bifunctional bis(thiosemicarbozone) ligands have been designed to improve the specificity of the copper deliv-

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ery.[45,46] Their potential interactions with copper-transport proteins should form part of further developments.

Acknowledgment. We thank the Australian Research Council for financial support.

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Supporting Information Available: Electrochemical data (Table S1), estimation of $K_D[Cu^I(Btsc)]$ (Table S2), principle and estimation of β_2 for $[Cu^I(Bca)_2]^{3-}$ (Tables S3−S5), and solution spectra (Figures S1−S6). This material is available free of charge via the Internet at http://pubs.acs.org.

IC702440E

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