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High Cu(I) and low proton affinities of the CXXC motif of *Bacillus subtilis* CopZ

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Running title: Cu(I) and proton affinities of CopZ

Keywords: copper trafficking, cysteine thiol, *Bacillus subtilis*, copper-mediated dimerization, β_2 formation constant

ABSTRACT: CopZ, an Atx1-like copper chaperone from the bacterium *Bacillus subtilis*, functions as part of a complex cellular machinery for Cu(I) trafficking and detoxification, in which it interacts specifically with the transmembrane Cu(I)-transporter CopA. Here we demonstrate that the cysteine residues of the MXCXXC Cu(I)-binding motif of CopZ have low proton affinities, with both exhibiting pK_a values at 6 or below. Chelator competition experiments demonstrated that the protein binds Cu(I) with extremely high affinity, with a small but significant pH-dependence over the range pH 6.5 – 8.0. From these data, a pH-corrected formation constant, $\beta_2 = \sim 6 \times 10^{22} \text{ M}^{-2}$, was determined. Rapid exchange of Cu(I) between CopZ and the Cu(I)-chelator BCS indicated that the mechanism of exchange does not involve simple dissociation of Cu(I) from CopZ (or BCS), but instead proceeds via the formation of a transient Cu(I)-mediated protein-chelator complex. Such a mechanism has similarities to the Cu(I)-exchange pathway that occurs between components of copper trafficking pathways.

INTRODUCTION

To satisfy the need for the incorporation of copper into proteins essential for processes such as respiration and photosynthesis [1], and to counter the potential toxicity of the metal ion, organisms have evolved complex mechanisms for trafficking copper (as Cu(I)) [2, 3]. These function to maintain Cu(I) in a tightly bound form, only releasing it through a specific facile interaction with target proteins, which are either copper-requiring enzymes or copper transporters.

The best conserved component of these pathways involves a small copper chaperone and a P-type ATPase transporter [4-7], which function in transmembrane Cu(I)-transport, and are found in all cell types. In humans, the chaperone Hah1 (Atox1) delivers Cu(I) to two P-type ATPase transporters, the Menkes' and Wilson's proteins, while in yeast the chaperone Atx1 delivers Cu(I) to the transporter CCC2 [3]. The Cu(I)-binding motif of copper chaperones (and, in fact, the N-terminal domain(s) of Cu(I)-transporting ATPases) feature the motif MXCXXC, in which the cysteine residues provide thiolate ligands to copper, stabilising it in the +1 oxidation state and preventing potentially hazardous redox activity.

Most bacteria also contain copper chaperone/transporter systems. In the Gram-positive bacterium *Bacillus subtilis*, CopZ and CopA, a copper chaperone and P-type ATPase, respectively, constitute a copper efflux system [8-12]. Significant structural and mechanistic information is now available for these proteins, [8-10, 13, 14] and it has been demonstrated that CopZ and the N-terminal part of CopA specifically interact [11, 15]. Previous studies of Cu(I)-binding to CopZ revealed that it undergoes Cu(I)-mediated dimerization, to form a $\text{Cu}(\text{CopZ})_2$ complex [9], as found for human Hah1 [16, 17]. Addition of further Cu(I) led to the formation of high order Cu(I)-bound forms of the protein, in which CopZ remained dimeric [9, 18].

Here we report detailed studies of Cu(I) binding to CopZ, in which competition experiments using the Cu(I)-chelators bathocuprione disulfonate (BCS) and bicinchoninic acid (BCA) demonstrated extremely tight binding of Cu(I). Measurements between pH 6.5 and 8.0 showed a small but significant pH dependence. Studies of the acid-base properties of apo-CopZ revealed that this dependence is not due to the binding motif cysteines, as both have pK_a values far below that of free cysteine. Kinetic studies showed that, in the presence of BCS, Cu(I)-dissociation from CopZ occurs rapidly, consistent with a Cu(I) exchange mechanism that involves the formation of a Cu(I)-mediated protein-chelator complex.

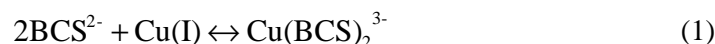
EXPERIMENTAL

Preparation of Cu(I)-CopZ species

Wild-type CopZ was purified as previously described [9]. Prior to the addition of Cu(I) ions, protein samples were treated with 5 mM DTT and excess reductant was removed by passage down a G25 Sephadex column (PD10, GE Healthcare) in an anaerobic glovebox (Faircrest), in which the oxygen concentration was kept below 2 ppm. Additions of Cu(I) were made anaerobically using a solution of CuCl in 1 M NaCl and 100 mM HCl [9, 19] or in 1 M NaCl and 10 mM HCl [17]; identical results were obtained in each case.

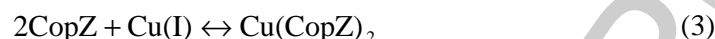
CopZ competition studies with Cu(I)-chelating ligands

Bathocuprione disulfonate (BCS) binds Cu(I) according to Equ. 1 and the overall formation constant, $\beta_{2(\text{BCS})}$, is given by Equ. 2.



$$\beta_{2(\text{BCS})} = \frac{[\text{Cu(BCS)}_2^{3-}]}{[\text{BCS}^{2-}]^2[\text{Cu}]} \quad (2)$$

$\beta_{2(\text{BCS})} = K_1K_2$, where K_1 is the association constant relating to the binding of the first BCS to Cu(I), and K_2 to the binding of the second. The binding of Cu(I) to CopZ and the corresponding overall formation constant can be written as in Equ. 3 and 4, respectively.



$$\beta_{2(\text{CopZ})} = \frac{[\text{Cu(CopZ)}_2]}{[\text{CopZ}]^2[\text{Cu}]} \quad (4)$$

The value of $\beta_{2(\text{CopZ})}$ can be estimated through Cu(I)-exchange between CopZ and BCS, Equ. 5, 6 and 7.



$$K_{ex} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu(CopZ)}_2][\text{BCS}^{2-}]^2}{[\text{Cu(BCS)}_2^{3-}][\text{CopZ}]^2} \quad (6)$$

$$\beta_{2(\text{CopZ})} = K_{ex}\beta_{2(\text{BCS})} \quad (7)$$

To determine the formation constant for the initial binding of Cu(I) to CopZ, experiments were carried out in both directions of Equ. (5) under anaerobic conditions with either CopZ and BCS pre-loaded with Cu(I). The concentration of CopZ was always in > 2-fold excess of Cu(I), to ensure that only the Cu(CopZ)₂ complex is formed (*ie* avoiding higher order Cu(I)-species). Following additions of either apo-CopZ or BCS, solutions were left for 10 min to reach equilibrium (at this time point, changes in $A_{483 \text{ nm}}$ were complete) and the absorption at 483 nm was recorded using a Jasco V550 spectrophotometer. Cu(BCS)₂³⁻ exhibits a strong absorption band centered at this wavelength [20] ($\epsilon_{483 \text{ nm}} = 13,300 \text{ M}^{-1} \text{ cm}^{-1}$). Data were corrected for dilution effects. $A_{483 \text{ nm}}$ values were used to calculate the final concentration of Cu(BCS)₂³⁻ in each solution. The exchange constant, K_{ex} , was determined from Equ. 6, and the formation constant for CopZ, $\beta_{2(\text{CopZ})}$, was subsequently calculated using Equ. 7 with $\beta_{2(\text{BCS})} = 6.3 \times 10^{19} \text{ M}^{-2}$ [20]. Note that identical results were obtained by calculating the concentration of free Cu(I) as previously described [20].

The formation constant for CopZ, $\beta_{2(\text{CopZ})}$, was also determined from equivalent competition experiments to those described above using another Cu(I)-chelator bicinchoninic acid (BCA). An overnight incubation was required in this case to ensure that equilibrium was reached. Cu(BCA)₂³⁻ exhibits a strong absorption band centered at 562 nm ($\epsilon_{562 \text{ nm}} = 7,700 \text{ M}^{-1} \text{ cm}^{-1}$), which was used to calculate the final concentration of Cu(BCA)₂³⁻ in each solution. The exchange constant, K_{ex} , was determined from the BCA equivalent of Equ. 6, and the formation constant for CopZ, $\beta_{2(\text{CopZ})}$, was subsequently calculated as in Equ. 7 with $\beta_{2(\text{BCA})} = 4.6 \times 10^{14} \text{ M}^{-2}$ [17]. We note that a K_1 constant for the initial binding of Cu(I) to chelator has been used in some cases to estimate the concentration of free Cu(I) [17]; however, for calculations of free metal ion concentrations, it is more usual to use the β_n formation constant [20-22] as K_1 alone does not accurately describe the equilibrium composition.

For studies of the pH dependence of Cu(I)-binding to CopZ, BCS and BCA competition experiments as above were carried out over a range of pH values. Equ. 8 and 9 were used to model one and two proton dependencies, respectively (see Supplementary Data).

$$K_{ex} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu}(\text{CopZ})_2][\text{BCS}^{2-}]^2(K + [\text{H}^+])^2}{[\text{Cu}(\text{BCS})_2^{3-}]K^2[\text{CopZ}]_{f,T}^2} \quad (8)$$

$$K_{ex} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu}(\text{CopZ})_2][\text{BCS}^{2-}]^2(1 + K_1[\text{H}^+] + \beta_{2,H}[\text{H}^+]^2)}{[\text{Cu}(\text{BCS})_2^{3-}][\text{CopZ}]_{f,T}^2} \quad (9)$$

where K is $1/K_a$ (*ie*, the reciprocal of the acid dissociation constant).

Determination of pK_a values of CopZ active site cysteines

The reaction of cysteine side chains with alkylating reagents is well established and occurs only with the ionized thiolate anion [23]. Measurement of the rate of alkylation as a function of pH can be used to determine the pK_a values of protein cysteine thiol groups [24-27], where the observed rate constant is proportional to the extent of thiol deprotonation at a given pH value [26]. CopZ was pre-reduced with 5 mM DTT and excess DTT removed via a Sephadex G25 column. CopZ (final conc. 1 μM) was added to a badan (Molecular Probes) solution (final conc. 13 μM) [28] in a mixed buffer system containing potassium acetate, Mes, Mops and Tris (10 mM each) and 200 mM KCl [26], and incubated for 2 h. Fluorescence spectra were recorded between 400 - 600 nm (ex. wavelength 391 nm) at 10 °C using a Perkin Elmer LS55 fluorescence spectrophotometer.

Fluorescence data were fitted to a single exponential function to obtain an observed, pseudo-first order rate constant (k_o). Where necessary, a double exponential fit was used and the rate constant for the initial reaction was taken as k_o . A pK_a value was determined by plotting k_o values as a function of pH and fitting to equation (10), which describes a single pK_a process, and where k_{SH} and k_{S^-} are the rate constants for the protonated and deprotonated forms, respectively [26].

$$k_o = \frac{k_{SH} + k_{S^-} 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \quad (10)$$

For MALDI-TOF experiments CopZ-badan reactions were carried out as described above. 10 mg ml⁻¹ alpha-cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% acetonitrile and 0.05% trifluoroacetic acid was used as the matrix. Spectra were recorded using a Shimadzu Biotech AXIMA-CFR instrument operating in linear mode, calibrated using cytochrome *c* (equine) 12,361.96 Da, apomyoglobin (equine) 16,952.27 Da and aldolase (rabbit muscle) 39,212.28 Da (Sigma).

RESULTS

Determination of the β_2 formation constant for $\text{Cu}(\text{CopZ})_2$ through competition studies with Cu(I)-chelators

Previously, it has been demonstrated that Cu(I) binding to CopZ is complex, resulting in the formation of a number of distinct species dependent on the Cu(I)/CopZ ratio. Initial binding of Cu(I) occurs from 0 - 0.5 Cu(I)/CopZ, leading to the dimerisation of the protein and formation of the complex $\text{Cu}(\text{CopZ})_2$ [9]. Cu(I) is likely to bridge the Cu(I)-binding motifs of the two monomers, in a similar arrangement to that structurally characterised for Hah1 [16].

Although it is known that initial Cu(I)-binding occurs with high affinity, this has not been quantitated. In order to determine very high affinities, use is often made of competitor ligands which have well characterized binding properties. For Cu(I), one such useful competitor ligand is bathocuproine disulfonic acid (BCS). Fig. 1A shows UV-visible absorption spectra following the addition of increasing concentrations of CopZ to $\text{Cu}(\text{BCS})_2^{3-}$. Inset is a plot of $\Delta A_{483 \text{ nm}}$ as a function of the ratio of CopZ to Cu(I). This clearly shows that the titration saturated at a level of just over 2 CopZ/Cu(I), corresponding to the complete formation of $\text{Cu}(\text{CopZ})_2$. Hence, in a competition in which CopZ was present at concentrations similar to that of BCS, a large majority of the Cu(I) was complexed by CopZ. This clearly indicated that the affinity of CopZ for Cu(I) is significantly higher than that of BCS. The reverse experiment, in which BCS was titrated into a solution of $\text{Cu}(\text{CopZ})_2$, demonstrated the formation of only low concentrations of $\text{Cu}(\text{BCS})_2^{3-}$ (note that to facilitate the

detection of the $\text{Cu}(\text{BCS})_2^{3-}$ complex the concentration of Cu(I) in this experiment was 2.5 greater than that present in the experiment described above). A plot of $A_{483 \text{ nm}}$ as a function of BCS/Cu(I) revealed a shallow linear absorbance increase at 483 nm (Fig. 1B). However, even at a concentration of BCS two-fold greater than that of CopZ, only ~10% of the Cu(I) was complexed by BCS, confirming that this ligand, under these conditions, cannot compete well with CopZ for Cu(I).

A series of experiments at much higher BCS concentrations were subsequently performed in order to determine the overall formation constant (β_2) for $\text{Cu}(\text{CopZ})_2$. From the absorbance measured at 483 nm, the concentration, at equilibrium, of $\text{Cu}(\text{BCS})_2^{3-}$ was determined, along with concentrations of $\text{Cu}(\text{CopZ})_2$, free CopZ and free BCS. From these, an exchange constant for the distribution of Cu(I) between BCS and CopZ was calculated as described in the Experimental section, see Table 1. The formation constant for $\text{Cu}(\text{BCS})_2^{3-}$ is known, $\beta_{2(\text{BCS})} = 6.3 \times 10^{19} \text{ M}^{-2}$ [20], and so the value of $\beta_{2(\text{CopZ})}$ was determined, Table 1.

Bicinchoninic acid (BCA) is another high affinity Cu(I)-chelator that has been used to determine stability constants for Cu(I)-binding to human Hah1 [17]. Experiments similar to those conducted with BCS were carried out with BCA (with absorbance at 562 nm used to calculate the concentration of $\text{Cu}(\text{BCA})_2^{3-}$) and the exchange constant for the distribution of Cu(I) between BCA and CopZ was calculated as above. The formation constant for $\text{Cu}(\text{BCA})_2^{3-}$ is known, $\beta_{2(\text{BCA})} = 4.6 \times 10^{14} \text{ M}^{-2}$ [17], and from this, the value of $\beta_{2(\text{CopZ})}$ was determined, Table 2. Good agreement was observed between BCS and BCA competition experiments: β_2 values were well within two orders of magnitude of each other, giving an overall average value of $\beta_{2(\text{CopZ})} = \sim 5.7 \times 10^{21} \text{ M}^{-2}$.

The pH dependence of Cu(I) binding to CopZ

The above analysis assumed that the binding of Cu(I) to CopZ is not subject to proton competition. This may not be the case because Cu(I) binding to CopZ involves the side chains of two cysteine residues present in the Cu(I)-binding motif [8, 9]. As Cu(I) binds only to the thiolate form of cysteine residues, the extent of Cu(I)-binding should be influenced by the pH of the experiment, which will dictate the extent to which H^+ ions can compete with Cu(I) for the thiolate groups.

Thus, the pH dependence of Cu(I) binding was investigated. Competition experiments with BCS and BCA were repeated at pH values of 6.5, 7.0, 7.5 and 8.0, see Tables 3 and 4 (complete details of these experiments are given in Supplementary Tables S1 and S2). Analysis of the data as above (Equ. 6) indicated that the K_{ex} values with both chelators exhibited a ~40 fold variation over this pH range. The expression for K_{ex} was, therefore, corrected to take account of the pH dependence. The rather shallow dependence is characteristic of the involvement of a single proton association/dissociation event, and the data for both chelators at pH 7 and above fitted well to Equ. 8, giving a $\text{p}K_a$ value of ~7.75, and an average corrected formation constant $\beta_{2(\text{CopZ})} = \sim 6 \times 10^{22} \text{ M}^{-2}$. At pH values much below 7, Cu(I)-binding to BCS and BCA becomes subject to proton competition (*eg* BCS $\text{p}K_a = 5.7$ [20]), contributing to a poorer fit of data at pH 6.5.

The small but significant dependence of the extent of Cu(I)-binding on pH raises the question of which group undergoes deprotonation/protonation with a $\text{p}K_a$ of ~7.75. Possibilities include the Cu(I)-binding motif cysteines or another, non-cysteine group that lies close enough to the active site to influence binding.

Determination of $\text{p}K_a$ values of the active site cysteines of CopZ

To determine whether the observed single-proton dependence is due to the protonation/deprotonation of a cysteine residue or perhaps another residue close to the binding motif, the $\text{p}K_a$ properties of the two Cu(I)-binding motif cysteine residues of CopZ (note that these are the only cysteine residues in *B. subtilis* CopZ) were investigated using the alkylating reagent 6-bromoacetyl-2-dimethylaminonaphthalene (badan), which forms a thioether bond with the side chain of cysteine residues, leading to a significant increase in its fluorescence intensity. The spectrum of unbound badan features a band at ~540 nm. On addition of CopZ the probe fluorescence showed a marked increase at 540 nm. The probe fluorescence is highly sensitive to its environment [28]; the cysteine residues undergoing modification in CopZ are clearly in a solvent exposed environment, consistent with structural data for CopZ [8].

Fluorescence at 540 nm was plotted against time at a range of pH values, see Fig. 2A. The initial increase and subsequent decrease of intensity observed at higher pH is most likely due to local unfolding/quenching caused by the addition of two labels at the active site [27]. At pH values above pH 6.5, this effect made it very difficult to obtain accurate rate information for the initial modification phase. Pseudo-first order rate constants were obtained from fits of the data at 540 and plotted against pH (for values below 6.5), Fig. 2B. The data fitted well to a single proton dissociation event, with a pK_a of 6.1 ± 0.1 .

MALDI-TOF spectra of CopZ samples reacted with badan at a range of pH values are shown in Fig. 2C. The spectrum of unreacted apo-CopZ contained a single peak at 7343 Da (which agrees well with the predicted mass of 7338.1 Da), while those of badan-reacted CopZ contained two further major peaks. Average values for the three species were 7340.3 ± 5 Da for unreacted, 7552.2 ± 8 Da for singly modified and 7764.2 ± 6 Da for doubly modified CopZ. The latter two correspond well with predicted masses of 7550.4 and 7762.7 Da for singly and doubly badan-modified CopZ, respectively. For the reaction at pH 4 the major peak corresponds to singly modified CopZ, clearly indicating that one of the CopZ active site cysteines has a pK_a below this pH value. As the pH was increased, the proportion of doubly labeled CopZ increased at the expense of unmodified and singly labeled proteins. The protein was essentially completely modified at pH 6 – 7, indicating that the second cysteine pK_a value is in this region. This is in good agreement with the badan fluorescence pK_a measurement (above) for which only a single deprotonation event with a pK_a value of 6.1 was identified. pH values lower than ~4 could not be explored because CopZ began to unfold at pH values below this (not shown).

Kinetic studies of Cu(I) transfer between CopZ and the chelator BCS

The BCS competition studies above demonstrate that CopZ has an extremely high affinity for Cu(I). Such high affinities imply that, because the on-rate for Cu(I)-binding to CopZ cannot be greater than that for a diffusion controlled reaction (second order rate constant $\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$), the off-rate (for dissociation of Cu(I) from CopZ) must be extremely low. However, the equilibrium competition studies carried out with BCS required only minutes to reach equilibrium (while those involving BCA required a significantly longer period to reach equilibrium). Therefore, BCS is not a passive reactant in the exchange reaction (and the same is true of CopZ in exchange reactions with $\text{Cu}(\text{BCS})_2^{3-}$), but instead must participate in facile metal ion exchange. A mechanism in which Cu(I) exchange between two molecules is facilitated by the formation of a Cu(I)-mediated hetero-complex is similar to that which occurs during transfer between copper chaperones and the N-terminal domains of Cu(I)-transporting ATPases [5, 16]. The nature of hetero-complexes formed, however, will be quite different, because BCS is a small molecule ligand and it coordinates Cu(I) through nitrogen ligands rather than cysteine side chains of the ATPase transporter.

To test the concentration dependence of the observed rate of the Cu(I)-exchange reaction, kinetic measurements of exchange were performed in both directions. Plots of $A_{483 \text{ nm}}$ as a function of time following additions of BCS to $\text{Cu}(\text{CopZ})_2$, and of CopZ to $\text{Cu}(\text{BCS})_2^{3-}$ are shown in Fig. 3. Final $A_{483 \text{ nm}}$ values were consistent with the thermodynamic data presented above. For the titration of $\text{Cu}(\text{CopZ})_2$ with BCS, each trace fitted well to a first order process (Fig. 3A). The first order rate constants obtained from the fits were plotted as a function of BCS concentration, Fig. 3C, demonstrating that the rate constant ($k = 0.019 \pm 0.002 \text{ s}^{-1}$ at 25 °C) was independent of the BCS concentration. Similarly, each trace from the titration of $\text{Cu}(\text{BCS})_2^{3-}$ with CopZ also fitted well to a single exponential (Fig. 3B) and a plot of rate constant as a function of CopZ concentration (Fig. 3C) showed that it has the same value as for the above reaction ($k = 0.019 \pm 0.002 \text{ s}^{-1}$ at 25 °C), and that it was also independent of the CopZ concentration.

DISCUSSION

The β_2 formation constant determined here for initial Cu(I)-binding to CopZ, resulting in the formation of the $\text{Cu}(\text{CopZ})_2$ complex, is the product of two individual equilibrium constants, K_1 and K_2 , corresponding to the binding of Cu(I) to a single CopZ and binding of a second CopZ, respectively. Previous studies indicated that at a level of 0.5 Cu/CopZ, a small proportion of monomeric protein is present [9], suggesting that K_2 is not particularly high, and we estimate that it is of the order of $\sim 1 \times$

$10^5 - \times 10^6 \text{ M}^{-1}$. Thus, K_1 must be extremely high, in the range $10^{17} - 10^{18} \text{ M}^{-1}$ (which can also be expressed as $K_d = 10^{-17} - 10^{-18} \text{ M}$, *ie* in the attomolar range), see Scheme 1.

Previous reports of Cu(I)-binding to proteins involved in Cu(I)-trafficking have revealed similarly high affinities. For example, CueR, the Cu(I)-responsive transcriptional regulator of *E. coli*, senses Cu(I) in the zeptomolar range [21, 29], while an association constant of $\sim 10^{18} \text{ M}^{-1}$ was reported for yeast Atx1 (determined by competition with BCS) [20]. Recent studies of human Hah1 (Atx1) revealed a K_1 of $3.5 \times 10^{10} \text{ M}^{-1}$ and a K_2 of $3.4 \times 10^6 \text{ M}^{-1}$ (for the formation of a Hah1-bridged Cu(I) dimer), with an overall β_2 of $1.2 \times 10^{17} \text{ M}^{-2}$ [17]. The latter was determined through competition experiments with BCA and it should be noted that a K_1 of $2.1 \times 10^7 \text{ M}^{-1}$ was used to determine the free Cu(I) concentration (whereas, for reasons discussed in the *Experimental* section we have used a β_2 value of $4.6 \times 10^{14} \text{ M}^{-2}$). Therefore, the work described here and that with Hah1 are not strictly comparable. However, it appears that CopZ has a significantly higher Cu(I) affinity than Hah1, as only $\sim 33\%$ of the Cu(I) present in a Cu(CopZ)₂ complex was given up to a ~ 230 -fold excess of BCA (when left at room temperature overnight). By contrast, a 45-fold excess of BCA was sufficient to remove 85% of Cu(I) from its complex with Hah1 within 1 hour at the same pH [17]. The tight binding of Cu(I) to CopZ determined here is consistent with the proposal that cells control copper toxicity by ensuring that they contain essentially no free copper (as demonstrated for yeast cells [30]).

The acid-base properties of the cysteine residue side chains of Cu(I)-binding motifs, have not, to our knowledge, been investigated previously. The data presented here demonstrate that both binding motif cysteine thiols exhibit low pK_a values, with one < 4 and the other ~ 6 (see Scheme 1). These are very significantly lower than that for free cysteine, which has a pK_a value of ~ 9 . The acid-base properties of thioredoxin-like proteins, which also contain a CXXC motif, have been extensively characterized and provide some useful comparisons. A very wide range of pK_a values are found in these proteins, from ~ 3.5 for the first cysteine of the active site motif of *E. coli* DsbA to ~ 8.8 for the same cysteine of *B. subtilis* ResA [26, 27]. For this family of proteins, there exists a direct relationship between the pK_a value of the first cysteine and the redox potential, with acidity correlated with a higher redox potential (*ie* stabilisation of the reduced state) [31-33]. In the case of copper chaperones, high acidity (low proton affinity) may also correlate with a high redox potential, which would protect the chaperone from oxidation to a disulfide bonded form. Low proton affinity of the binding motif cysteines may also correlate with high Cu(I) affinity.

Why are the CopZ cysteine thiol groups so acidic? Further useful comparisons can be made with thioredoxin-like proteins for which a very significant literature exists on the analysis of pK_a values of active site cysteines. In these proteins, an important contribution to the low pK_a values observed is the location of the active site at the N-terminus of an α -helix, such that it is optimally positioned to interact favourably with the helix dipole [34]. The CopZ active site is also located at the N-terminus of an α -helix, and so a similarly favourable interaction may be anticipated. Furthermore, in DsbA, which exhibits the lowest pK_a values (at least for the N-terminal cysteine), a histidine residue is located at position 2 of the dipeptide sequence separating the two active site cysteines. This is believed to interact electrostatically with the N-terminal cysteine thiolate [35], providing stabilization that results in a low pK_a value. CopZ has a histidine at the equivalent position. Ultimately, the pK_a is determined by the extent to which the thiolate form is stabilized (relative to the thiol form). In DsbA, high resolution structural data revealed several stabilizing hydrogen bond interactions [36], while in other thioredoxin-like proteins, in which pK_a values are higher, there are far fewer stabilizing interactions [37]. In CopZ and other Atx1-like chaperones, the cysteine thiols are exposed to the solvent, and so H-bonding interactions with water molecules could contribute to stabilization of the deprotonated form.

The observed pH dependence of Cu(I)-binding to CopZ, therefore, does not arise from proton competition at the ligating cysteine residues (because these are deprotonated in the pH range for which Cu(I) affinity was measured). Thus, it must be due to a protonation/deprotonation event involving a residue, close to the Cu(I)-binding motif, which influences the Cu(I)-binding affinity. One possibility is His15, which lies between the two binding motif cysteines. Protonation of this residue would be expected to inhibit binding of a positively charged metal ion close by. This residue is highly conserved amongst copper chaperones from Gram-positive bacteria, including *Bacillus* and related species, and the *actinomycetales* (including Streptomycetes and Mycobacteria), and also occurs in some Gram-negative proteobacteria, consistent with an important physiological function(s).

The kinetic measurements of Cu(I)-exchange rates presented here demonstrate that the exchange mechanism does not involve the dissociation of Cu(I) from CopZ and then chelation of the free Cu(I) by BCS (or BCA). Because of the very high affinity of CopZ for Cu(I), dissociation of this type would be extremely slow. Therefore, the small molecule chelator must play an active role in the exchange mechanism; that different exchange kinetics were observed for BCS and BCA is consistent with this. The kinetics of the BCS exchange reaction were probed in more detail. The data revealed that the rate of exchange from Cu(CopZ)₂ to BCS is independent of the concentrations of BCS (and that from Cu(BCS)₂ to CopZ is independent of the concentration of CopZ). Therefore, the rate-determining steps of the exchange reaction in both directions are true first order processes, and cannot correspond to the initial binding of BCS to Cu(CopZ)₂ or of CopZ to Cu(BCS)₂³⁻ (which are second order processes), implying the hetero-coordination of Cu(I) by CopZ and BCS occurs in a step subsequent to initial binding, see Scheme 1.

One possibility is that the rate determining steps correspond to the dissociation of Cu(CopZ)₂ to CuCopZ and CopZ (for the CopZ to BCS direction of transfer) and to the dissociation of Cu(BCS)₂ to CuBCS and BCS (for the BCS to CopZ direction), which are both first order processes, see Scheme 1. However, the data in Fig. 3C demonstrate that the rate constants are the same for both directions of the exchange reaction. It is highly unlikely that dissociation of Cu(CopZ)₂ and Cu(BCS)₂ occur with the same rate constant and, therefore, it is unlikely that these processes are the rate determining steps of the exchange reaction. Another possibility, and one that we favour, is that the rate determining step corresponds to the formation or breakdown of a CopZ-Cu(I)-BCS intermediate. This conclusion is supported by the fact that the apparent rate constant for the exchange reaction is independent of the direction of the exchange (Fig. 3C), indicating that a similar process is rate determining in each case, and therefore likely features the same intermediate species. We note that similarly rapid exchange of Cu(I) between BCS and yeast Atx1 was also observed [20]. The formation of a Cu(I)-mediated hetero-complex also occurs during the facile exchange of Cu(I) between Atx1-like chaperones and their cognate Cu(I)-transporting P-type ATPases, although the nature of the protein-protein complexes formed are clearly different to those of the protein-small molecule complex formed here. Scheme 1 provides an overview of proton and Cu(I)-binding to CopZ, and illustrates the proposed mechanism for bi-directional exchange of Cu(I) between CopZ and BCS.

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ABBREVIATIONS

Abbreviations used: Badan; 6-bromoacetyl-2-dimethylaminonaphthalene; DTT, dithiothreitol; MALDI-TOF, Matrix-assisted laser desorption/ionization-Time of Flight; Mes, 2-(N-morpholino)ethanesulfonate; Mops, 3-morpholinopropanesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

FIGURE LEGENDS

Figure 1. Investigation of the affinity of CopZ for Cu(I) through competition experiments with the chelator BCS. (A) UV-visible spectra following additions of apo-CopZ to a solution of $3.7 \mu\text{M}$ $\text{Cu}(\text{BCS})_2^{3-}$. Inset is a plot of $A_{483 \text{ nm}}$ as a function of the ratio of CopZ to total Cu(I). The stoichiometry of the reaction is indicated by the intersection of lines through the data points at the beginning and end of the titration. (B) UV-visible spectra following additions of BCS to $14.5 \mu\text{M}$ $\text{Cu}(\text{CopZ})_2$. Inset is a plot of $A_{483 \text{ nm}}$ as a function of the ratio of BCS to total Cu(I). No end point is observed in the titration. Both CopZ and BCS were in 100 mM Mops, 100 mM NaCl, pH 7.5. Arrows indicate the direction of absorbance changes.

Figure 2. Determination of the pK_a properties of the Cu(I)-binding motif cysteine thiols of CopZ. (A) Fluorescence intensity at 540 nm as a function of time following addition of apo-CopZ ($1 \mu\text{M}$) to badan ($13 \mu\text{M}$) at 10°C at different pH values, as indicated. Solid lines through the data represent a fit to an exponential function from which an observed, pseudo-first order rate constant, k_o , was obtained. (B) Plot of k_o as a function of pH. The solid line shows a fit to Equ. (10). (C) MALDI mass spectra of samples of apo-CopZ (at pH 7) before and following the addition of apo-CopZ ($1 \mu\text{M}$) to badan ($13 \mu\text{M}$) at different pH values, as indicated.

Figure 3. Kinetic studies of Cu(I) exchange between CopZ and BCS. (A) Plots $A_{483 \text{ nm}}$, reporting on $\text{Cu}(\text{BCS})_2^{3-}$ formation, following the addition of BCS (final concentration as indicated) to CopZ ($30 \mu\text{M}$) containing $10 \mu\text{M}$ Cu(I). (B) Plots of $\Delta A_{483 \text{ nm}}$, reporting on loss of $\text{Cu}(\text{BCS})_2^{3-}$, following the addition of apo-CopZ (final concentration as indicated). The solid lines through the data result from fits of each trace to a single exponential function. (C) Plot of first order rate constants (k), derived from fitting the data in (A) and (B) and similar experiments, as a function of BCS (lower abscissa) and CopZ (upper abscissa). CopZ and BCS were in 100 mM Mops, 100 mM NaCl, pH 7.5.

SCHEME LEGENDS

Scheme 1. Proton and Cu(I) binding to CopZ and the mechanism of Cu(I) exchange between CopZ and BCS. Proton dissociation from the thiol side chains of cysteines in the Cu(I)-binding motif and the stepwise formation of the $\text{Cu}(\text{CopZ})_2$ complex ($\beta_2 = \sim 6 \times 10^{22} \text{ M}^{-2}$) are illustrated. The relative sizes of CopZ and BCS are not to scale and it should be noted that BCS is illustrated schematically only; the complete structure of BCS (and BCA) is given in Fig. S1. To improve clarity, negative and positive charges associated with cysteine thiolates and Cu(I), respectively, are not shown in protein-metal complexes. The nature of the initial complex formed between CopZ and BCS (in which there is no hetero-Cu(I) chelation) is unknown and nothing should be inferred from the schematic representation of it. Reactions which could be rate-determining are indicated.

TABLES

Table 1. Formation constant, $\beta_{2(\text{CopZ})}$, for Cu(I)-binding to CopZ based on competition with BCS.

[P] _t , μM	29.0	30.0 ^a	29.0	30.0	29.0
[BCS] _t , mM	0.8	0.66	0.6	0.44	0.4
[Cu] _t , μM	9.6	10	9.4	10	9.2
[Cu(BCS) ₂ ³⁻], μM	8.41	8.41	7.01	6.58	5.15
[Cu(CopZ) ₂], μM	1.20	1.59	2.39	3.42	4.05
K_{ex} ^b	124	109	199	176	273
$\beta_{2(\text{CopZ})}$, M ⁻² ^c	7.8×10^{21}	6.9×10^{21}	1.26×10^{22}	1.11×10^{22}	1.72×10^{22}
Average $\beta_{2(\text{CopZ})}$, M ⁻²	$1.11 \pm 0.41 \times 10^{22}$				

^a The columns highlighted in grey are data resulting from addition of BCS to CopZ containing 0.3 Cu(I)/protein; those in white contain data from experiments in which CopZ was added to BCS solutions containing 10 μM Cu(I). Buffer was 100 mM Mops, 100 mM NaCl, pH 7.5.

^b From Equ. 6.

^c Calculation based on $\beta_{2(\text{BCS})} = 6.3 \times 10^{19} \text{ M}^{-2}$ for [Cu(BCS)₂]³⁻ (see the *Experimental* section).

Table 2. Formation constant, $\beta_{2(\text{CopZ})}$, for Cu(I)-binding to CopZ based on competition with BCA.

[P] _t , μM	30	30 ^a	30	30	30	30
[BCA] _t , mM	3.75	4.80	5.00	5.90	6.50	7.00
[Cu] _t , μM	10.0	10.0	10.0	10.0	10.0	10.0
[Cu(BCA) ₂ ³⁻], μM	1.41	1.32	2.66	1.43	2.50	3.25
[Cu(CopZ) ₂], μM	8.59	8.68	7.44	8.57	7.50	6.75
K_{ex} ($\times 10^5$) ^b	5.2	4.9	2.9	7.1	5.6	5.6
$\beta_{2(\text{CopZ})}$, M^{-2c}	2.40×10^{20}	2.25×10^{20}	1.35×10^{20}	3.27×10^{20}	2.59×10^{20}	2.58×10^{20}
Average $\beta_{2(\text{CopZ})}$, M^{-2}	$2.41 \pm 0.62 \times 10^{20}$					

^a The columns highlighted in grey are data resulting from addition of BCA to CopZ containing 0.3 Cu(I)/protein; those in white contain data from experiments in which CopZ was added to BCA solutions containing 10 μM Cu(I). Buffer was 100 mM Mops, 100 mM NaCl, pH 7.5.

^b From the BCA equivalent of Equ. 6.

^c Calculation based on $\beta_{2(\text{BCA})} = 4.6 \times 10^{14} \text{M}^{-2}$ for [Cu(BCA)₂]³⁻ (see the *Experimental* section).

Table 3. Correction of $\beta_{2(\text{CopZ})}$ through the pH dependence of K_{ex} between CopZ and BCS.

	pH 6.5	pH 7.0	pH 7.5	pH 8.0
[P] _t , μM	30.0 ^a	30.0	30.0	30.0
[BCS] _t , mM	0.22	0.44	0.44	0.44
[Cu] _t , μM	10.0	10.0	10.0	10.0
[Cu(BCS) ₂ ³⁻], μM	7.21	8.55	6.29	4.41
[Cu(CopZ) ₂], μM	2.79	1.45	3.71	5.59
K_{ex} ^b	27.4	41.4	211	667
pH corrected K_{ex} ^c	9740	1820	1630	1630
$\beta_{2(\text{CopZ})}$, M^{-2} ^d	5.1×10^{23}	1.16×10^{23}	0.96×10^{23}	1.30×10^{23}
Average $\beta_{2(\text{CopZ})}$, M^{-2} ^e	$1.25 \pm 0.54 \times 10^{23}$			

^a data result from addition of BCS to CopZ containing 0.3 Cu(I)/protein (see Table S1 for a complete data set). The buffer was 100 mM Mops, 100 mM NaCl at the pH values indicated.

^b From Equ. 6.

^c The pH correction of K_{ex} was obtained using a $\text{p}K_{\text{a}}$ of 7.75 resulting from fitting with Equ. 8 (see main text).

^d Calculation based on $\beta_{2(\text{BCS})} = 6.3 \times 10^{19} \text{M}^{-2}$ for [Cu(BCS)₂]³⁻ (see the *Experimental* section) using an average value of pH corrected K_{ex} , obtained from Table S1.

^e Average of data at pH 7 – 8.

Table 4. Correction of $\beta_{2(\text{CopZ})}$ through the pH dependence of K_{ex} between CopZ and BCA.

	pH 6.5	pH 7.0	pH 7.5	pH 8.0
[P] _t , μM	30.0	30.0	30.0	30.0
[BCA] _t , mM	4.76	4.76	4.76	4.76
[Cu] _t , μM	10.0	10.0	10.0	10.0
[Cu(BCA) ₂ ³⁻], μM	5.30	4.80	1.32	0.88
[Cu(CopZ) ₂], μM	4.70	5.20	8.68	9.12
K_{ex} ($\times 10^5$) ^b	0.47	0.64	9.32	16.9
pH corrected K_{ex} ($\times 10^6$) ^c	16.6	2.79	7.18	3.78
$\beta_{2(\text{CopZ})} \text{M}^{-2}$ ^d	7.35×10^{21}	1.83×10^{21}	3.30×10^{21}	1.74×10^{21}
Average $\beta_{2(\text{CopZ})} \text{M}^{-2}$ ^e	$2.26 \pm 1.00 \times 10^{21}$			

^a data result from addition of BCS to CopZ containing 0.3 Cu(I)/protein (see Table S2 for a complete data set). The buffer was 100 mM Mops, 100 mM NaCl at the pH values indicated.

^b From Equ. 6.

^c The pH correction of K_{ex} was obtained using a pK_{a} of 7.75 resulting from fitting with Equ. 8 (see main text).

^d Calculation based on $\beta_{2(\text{BCA})} = 4.6 \times 10^{14} \text{M}^{-2}$ for [Cu(BCA)₂]³⁻ (see the *Experimental* section) using an average value of pH corrected K_{ex} , obtained from the data in Table S2.

^e Average of data at pH 7 – 8.

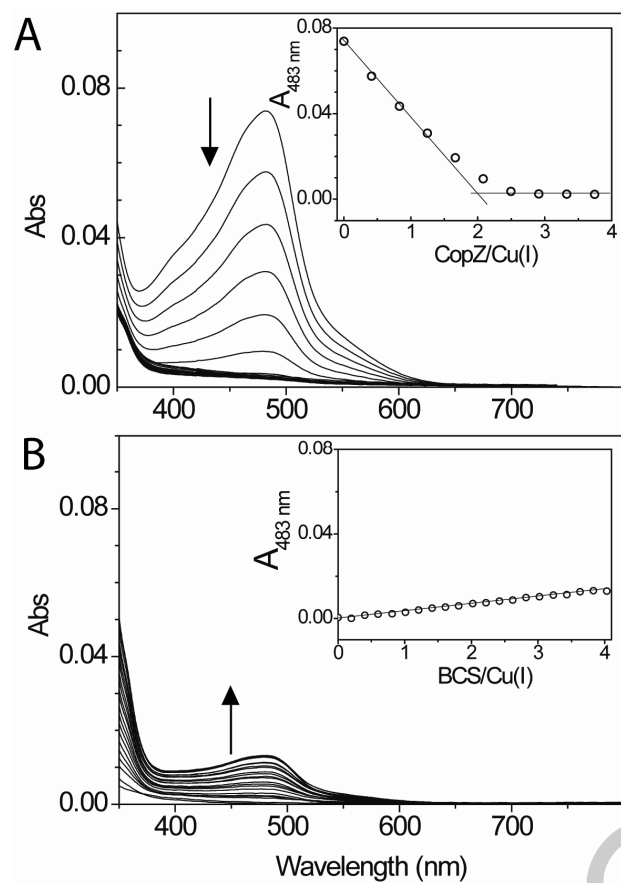


Fig. 1

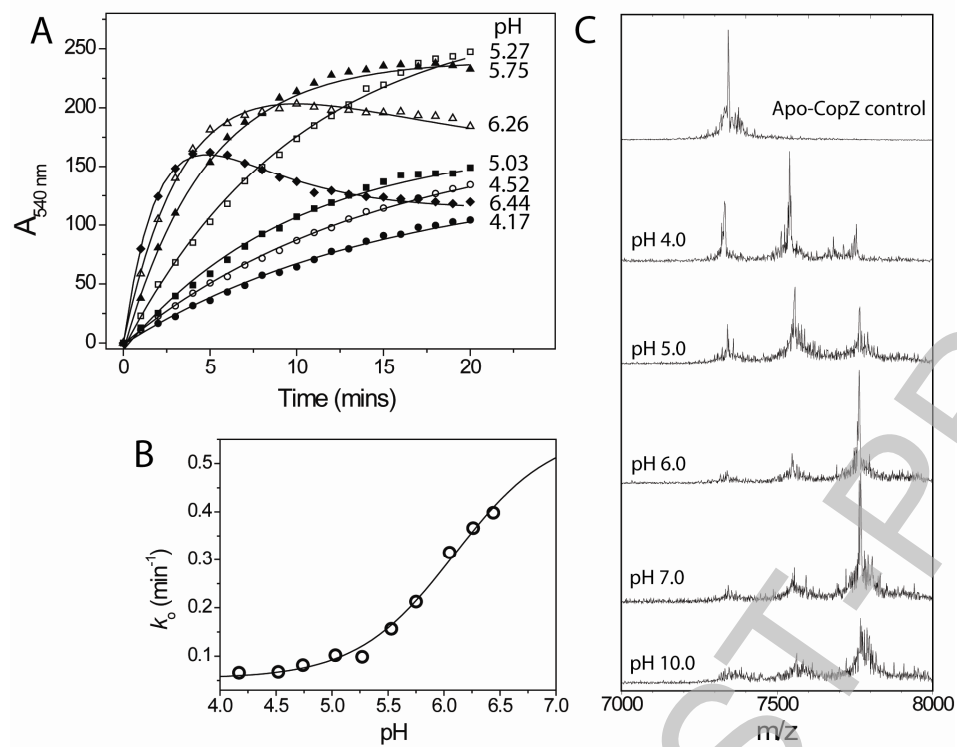


Fig. 2.

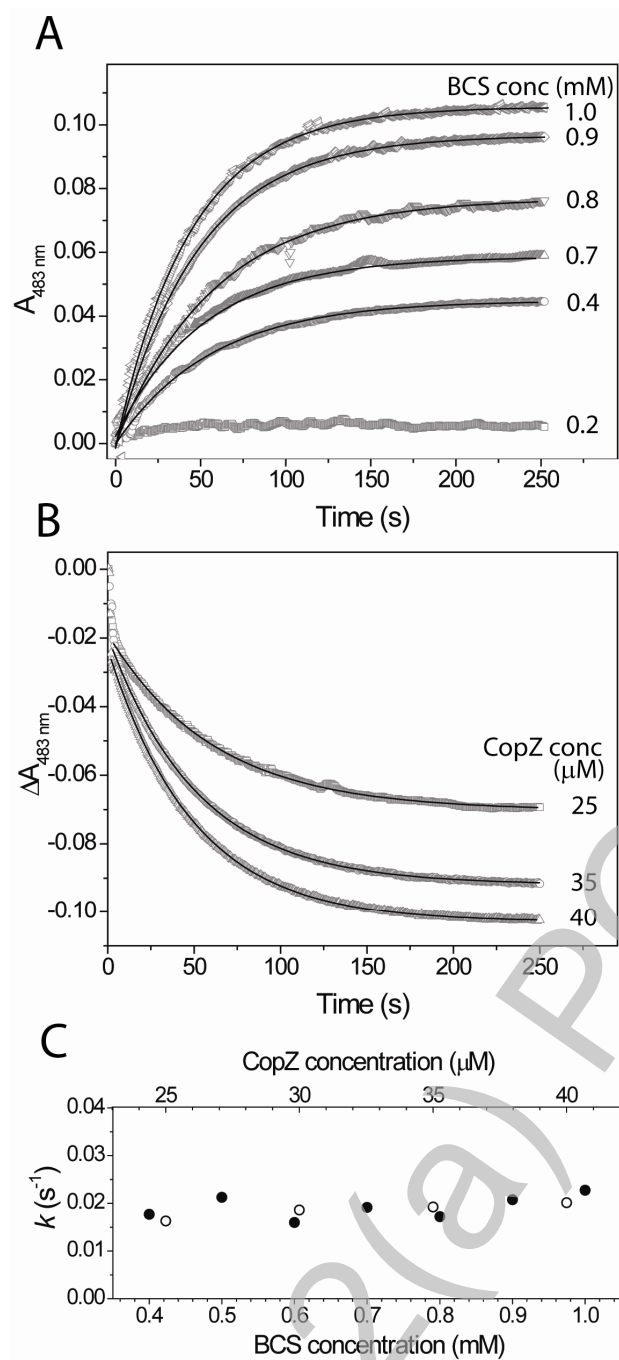
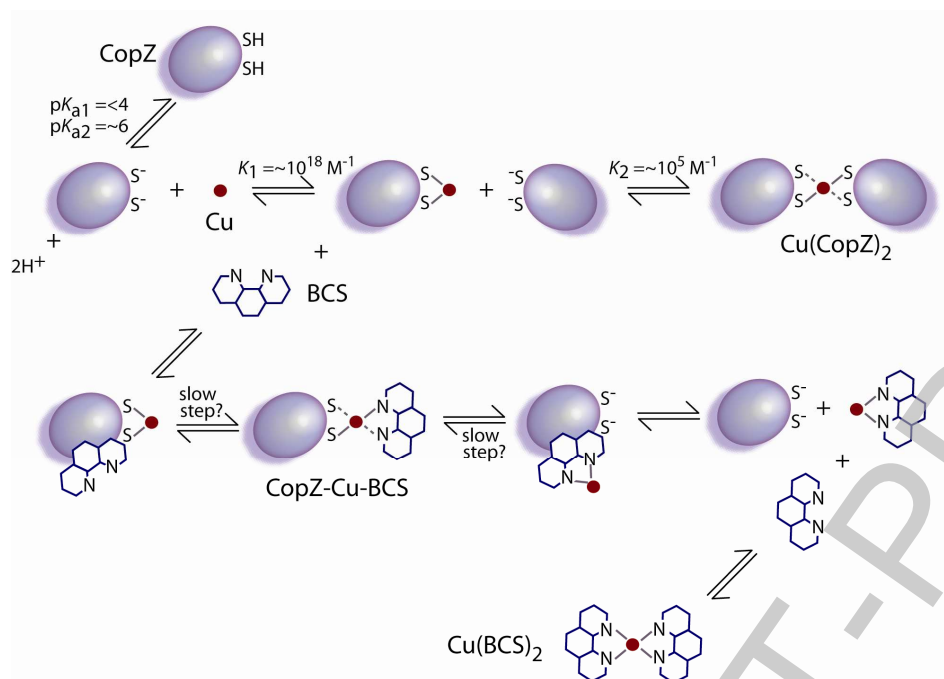


Fig. 3



Scheme 1.