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

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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 of 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, $\beta_1 = 6 \times 10^{25}$ M$^{-2}$, was determined. Rapid exchange of Cu(I) between COPZ and the Cu(I)-chelator BCS (bathocuprine disulfonate) 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.

Key words: Bacillus subtilis, copper trafficking, copper-mediated dimerization, cysteine thiol, $\beta_1$ formation constant.

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 (Atx1) 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, stabilizing 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 CuCopZ2 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 BCS (bathocuprine disulfonate) and BCA (bicinchoninic acid) 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 (dithiothreitol) 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 p.p.m. 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

BCS binds Cu(I) according to eqn (1), and the overall formation constant, $\beta_{2(BCS)}$, is given by eqn (2):

$$2\text{BCS}^{2-} + \text{Cu(I)} \leftrightarrow \text{Cu(BCS)}^{+}$$

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

$\beta_{2(BCS)} = K_1 K_2$, 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 eqns (3) and (4), respectively:

$$2\text{CopZ} + \text{Cu(I)} \leftrightarrow \text{Cu(CopZ)}_2$$  \hspace{1cm} (3)

$$\beta_{2(\text{CopZ})} = \frac{[\text{Cu(CopZ)}_2]}{[\text{CopZ}][\text{Cu}]}$$  \hspace{1cm} (4)

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

$$\text{Cu(CopZ)}_2 + 2\text{BCS}^{2-} \leftrightarrow 2\text{CopZ} + \text{Cu(BCS)}_3^{3-}$$  \hspace{1cm} (5)

$$K_{\text{ex}} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu(CopZ)}_2][\text{BCS}^{2-}]^2}{[\text{Cu(BCS)}_3^{3-}][\text{CopZ}]^2}$$  \hspace{1cm} (6)

$$\beta_{2(\text{BCS})} = K_{\text{ex}}\beta_{2(\text{BCS})}$$  \hspace{1cm} (7)

where $K_{\text{ex}}$ is the exchange constant. To determine the formation constant for the initial binding of Cu(I) to CopZ, experiments were carried out in both directions of eqn (5) under anaerobic conditions with either CopZ or 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 [i.e. 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 $A_{\lambda_{433}}$ were complete) and the absorption at 483 nm was recorded using a Jasco V550 spectrophotometer. Cu(BCS)$_3^{3-}$ exhibits a strong absorption band centred at this wavelength [20] ($\varepsilon_{433\text{nm}} = 13.300 \text{ M}^{-1}\cdot\text{cm}^{-1}$). Results were corrected for dilution effects. $A_{\lambda_{433}}$ values were used to calculate the final concentration of Cu(BCS)$_3^{3-}$ in each solution. The exchange constant, $K_{\text{ex}}$, was determined from eqn (6), and the formation constant for CopZ, $\beta_{2(\text{CopZ})}$, was subsequently calculated using eqn (7), with $\beta_{2(\text{BCS})} = 6.3 \times 10^{10} \text{ M}^{-2}$ [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 competition experiments equivalent to those described above using another Cu(I) chelator, BCA. An overnight incubation was required in this case to ensure that equilibrium was reached. Cu(BCA)$_3^{3-}$ exhibits a strong absorption band centred at 562 nm ($\varepsilon_{562} = 7700 \text{ M}^{-1}\cdot\text{cm}^{-1}$), which was used to calculate the final concentration of Cu(BCA)$_3^{3-}$ in each solution. The exchange constant, $K_{\text{ex}}$, was determined from the BCA equivalent of eqn (6), and the formation constant for CopZ, $\beta_{2(\text{CopZ})}$, was subsequently calculated as in eqn (7) with $\beta_{2(\text{BCS})} = 4.6 \times 10^{14} \text{ M}^{-2}$ [17]. We note that a $K_{\text{f}}$ 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 $\beta_{2}$ formation constant [20–22], as $K_{\text{f}}$ 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. Eqns (8) and (9) were used to model one- and two-proton deprotonations respectively (see Supplementary Data at http://www.BiochemJ.org/bj/413/bj4130459add.htm):

$$K_{\text{ex}} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu(CopZ)}_2][\text{BCS}^{2-}]^2(K + [H^+]^2)}{[\text{Cu(BCS)}_3^{3-}][K][\text{CopZ}]_T^2}$$  \hspace{1cm} (8)

$$K_{\text{ex}} = \frac{\beta_{2(\text{CopZ})}}{\beta_{2(\text{BCS})}} = \frac{[\text{Cu(CopZ)}_2][\text{BCS}^{2-}]^2(1 + K][H^+] + \beta_{2(\text{H}^+)}[H^+]^2)}{[\text{Cu(BCS)}_3^{3-}][K][\text{CopZ}]_T^2}$$  \hspace{1cm} (9)

where $K$ is $1/K_a$ (i.e. the reciprocal of the acid dissociation constant) and $[\text{CopZ}]_T$ is the total concentration of free CopZ.

### Determination of $\beta_{2}$ 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 [21]. Measurement of the rate of alkylation as a function of pH can be used to determine the $\beta_{2}$ 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 was removed via a Sephadex G25 column. CopZ (final concentration 1 μM) was added to a badan (6-bromoacetyl-2-dimethylaminonaphthalene; Molecular Probes) solution (final concentration 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 (excitation 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 $k_s$. A $\beta_{2}$ value was determined by plotting $k_o$ values as a function of pH and fitting to eqn (10), which describes a single $\beta_{2}$ process, and where $k_s$, $k_s$, and $k_s$ are the rate constants for the protonated and deprotonated forms respectively [26]:

$$k_o = \frac{k_s + k_s \cdot 10^{pK_a - pK_s}}{1 + 10^{pK_a - pK_s}} \hspace{1cm} (10)$$

For MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) experiments, CopZ/badan reactions were carried out as described above. CHCA (α-cyano-4-hydroxycinnamic acid; 10 mg·ml$^{-1}$), 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) 12361.96 Da, apomyoglobin (equine) 16952.27 Da and aldolase (rabbit muscle) 39212.28 Da (Sigma).

### RESULTS

### Determination of the $\beta_{2}$ formation constant for Cu(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 dimerization of the protein and formation of the complex Cu(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 characterized for Hah1 [16]. Although it is known that initial Cu(I) binding occurs with high affinity, this has not been quantified. 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 BCS. Figure 1(A) shows UV/visible absorption spectra following the addition of increasing concentrations of CopZ to Cu(BCS)$_3^{3-}$. Inset is a plot of $\Delta A_{\lambda_{433}}$ 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 Cu(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 Cu(CopZ)₂, demonstrated the formation of only low concentrations of Cu(BCS)₃⁻ [note that to facilitate the detection of the Cu(BCS)₃⁻ complex, the concentration of Cu(I) in this experiment was 2.5 times greater than that present in the experiment described above]. A plot of A₄₈₃ as a function of BCS/Cu(I) revealed a shallow linear absorbance increase at 483 nm (Figure 1B). However, even at a concentration of BCS/Cu(I) 2.5 times greater than that present in the experiment described above, a plot of A₄₈₃ as a function of BCS/Cu(I) revealed a shallow linear absorbance increase at 483 nm (Figure 1B). However, even at a concentration of BCS/Cu(I) revealed a shallow linear absorbance increase at 483 nm (Figure 1B).

A series of experiments at much higher BCS concentrations were subsequently performed in order to determine the overall 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 solution, 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 at http://www.BiochemJ.org/bj/413/bj4130459add.htm). Analysis of the data as above [eqn (6)] indicated that the Kₐ values with both chelators exhibited a 40-fold variation over this pH range. The expression for Kₐ was, therefore, corrected to take account of the pH dependence. The rather small 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 eqn (8), giving a pKₐ value of ~7.75, and an averaged corrected formation constant β₂(CopZ) = ~6 × 10²² M⁻². At pH values below 7, Cu(I) binding to BCS and BCA becomes subject to proton competition (e.g. BCS pKₐ = 5.7 [20]), contributing to a poorer fit of data at pH 6.5.
Table 2 Formation constant, \( \beta_{(BCA)} \), for Cu(I) binding to CopZ based on competition with BCA

Comparison of data resulting from addition of BCA to CopZ containing 0.3 Cu(I)/protein with data from experiments in which CopZ was added to BCA solutions containing 10 \( \mu \)M Cu(I). The buffer used was 100 mM Mops and 100 mM NaCl, pH 7.5. *From the BCA equivalent of eqn (6). \( \beta_{(BCA)} \) is obtained from fits of the data at 540 nm and plotted against pH. The data fitted well to a determination of \( \beta_{(BCA)} \) for which only a single deprotonation event with a pK\(a \) of 7.75. \( \beta_{(BCA)} \) values of the active site cysteines of CopZ

Table 3 Correction of \( \beta_{(BCA)} \) through the pH dependence of K\(a \) between CopZ and BCS

Table 4 Correction of \( \beta_{(BCA)} \) through the pH dependence of K\(a \) between CopZ and BCA

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 pK\(a \) of \( \sim 7.75 \). 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 \( \beta_{(BCA)} \) 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 pK\(a \)s 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 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 \( \sim 540 \) 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 Figure 2(A). The initial increase and subsequent decrease of intensity observed at higher pH values 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 nm and plotted against pH (for values below 6.5) in Figure 2(B). The data fitted well to a single proton dissociation event, with a pK\(a \) of 6.1 \( \pm \) 0.1.

MALDI–TOF spectra of CopZ samples reacted with badan at a range of pH values are shown in Figure 2(C). 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 that of badan-reacted CopZ contained two further major peaks. Average values for the three species were 7340.3 \( \pm \) 5 Da for unreacted, 7552.2 \( \pm \) 8 Da for singly modified and 7764.2 \( \pm \) 6 Da for doubly modified CopZ. The last 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-labelled CopZ increased at the expense of unmodified and singly-labelled 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 $\sim 4$ could not be explored because CopZ began to unfold at pH values below this (results 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 $\sim 10^10 \text{ M}^{-1} \cdot \text{s}^{-1}$), 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 Cu(CpZ)$_2^-$], 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 the 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 $A_{483}$ as a function of time following additions of BCS to Cu(CopZ)$_2$, and of CopZ to Cu(BCS)$_2^-$ are shown in Figure 3. Final $A_{483}$ values were consistent with the thermodynamic data presented above. For the titration of Cu(CopZ)$_2$ with BCS, each trace fitted well to a first-order process (Figure 3A). The first-order rate constants obtained from the fits were plotted as a function of BCS concentration (Figure 3B), 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 Cu(BCS)$_2^-$ with CopZ also fitted well to a single exponential (Figure 3C), and a plot of rate constant as a function of CopZ concentration (Figure 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 $\beta_2$, formation constant determined here for initial Cu(I) binding to CopZ, resulting in the formation of the Cu(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 $\sim 1 \times 10^8 \times 10^9 \text{ M}^{-1}$. Thus $K_2$ must be extremely high, in the range $10^{17}$–$10^{18} \text{ M}^{-1}$ (which can also be expressed as $K_d = 10^{-17}–10^{-18} \text{ M}$, i.e. 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 *Escherichia coli*, senses Cu(I) in the zeptomolar range [21,29], while an association constant of $\sim 10^{10} \text{ M}^{-1}$ 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 $3.4 \times 10^9 \text{ M}^{-1}$ [for the formation of a Hah1-bridged Cu(I) dimer], with an overall $\beta_2$ of $2.3 \times 10^{-17} \text{ M}^{-2}$ [17]. The latter was determined through competition experiments with BCA, and it should be noted that a $K_1$ of $2.1 \times 10^9 \text{ M}^{-1}$ was used to determine the free Cu(I) concentration (whereas, for reasons discussed in the Experimental section, we have used a $\beta_2$ 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 $\sim 230$-fold excess of BCA (when left at room temperature, 20°C, overnight). By contrast, a 45-fold excess of BCA was
The CopZ active site is also located at the N-terminus of an 
allo position to interact favourably with the helix dipole [34].

Why are the CopZ cysteine thiol groups so acidic? Further useful comparisons can be made with thioredoxin-like proteins, for which some very significant literature exists on the analysis of pKₐ values of active site cysteines. In these proteins, an important contribution to the low pKₐ 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 pKₐ 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 pKₐ value. CopZ has a histidine at the equivalent position. Ultimately, the pKₐ 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 pKₐ values are higher, there are far fewer stabilizing interactions [37]. In CopZ and other Atx1-like chaperones, the cysteine thios 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 His⁵⁵, 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 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 results revealed that the rate of exchange from Cu(CopZ)₂ to BCS is independent of the concentrations of BCS [and that from Cu(CopZ)₃ 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)₃⁻ (which are second-order processes), implying the hetero-co-ordination 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)₃ to CuCopZ₂ and CuCopZ (for the CopZ to BCS direction of transfer) and to the dissociation of Cu(BCS)₃⁻ to BCS⁻ and BCS (for the BCS to CopZ direction), which are both first-order processes; see Scheme 1. However, the data in Figure 3(C) demonstrate that the rate constants are the same for both directions of the exchange reaction. It is highly unlikely that dissociation of Cu(CopZ)₃ and Cu(BCS)₃ occurs 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 (Figure 3C), indicating that a similar process is rate determining in each case, and therefore probably features the same intermediate species. We note that a similarly rapid exchange of Cu(I) between BCS and yeast Atx1 was also observed [20]. The formation of a

![Scheme 1 Proton and Cu(I) binding to CopZ and the mechanism of Cu(I) exchange between CopZ and BCS](http://www.BiochemJ.org/bj/413/bj4130459add.htm)
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 from 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.

This work was supported by the U.K.’s BBSRC (Biotechnology and Biological Sciences Research Council) through the award of a Studentship to C.S., by UEA (University of East Anglia) and the School of Chemical Sciences and Pharmacy through the award of financial support to I.L.Z., and by a Wellcome Trust award from the Joint Infrastructure Fund for equipment. We thank Dr Allison Lewin for assistance with pHK measurements.

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Received 28 February 2008; accepted 17 April 2008
Published as BJ Immediate Publication 17 April 2008, doi:10.1042/BJ20080467

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