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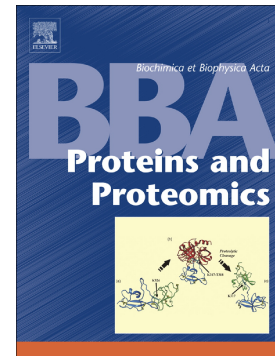
The N-terminal domains of Bacillus subtilis CopA do not form a stable complex in the absence of their inter-domain linker

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The N-terminal domains of *Bacillus subtilis* CopA do not form a stable complex in the absence of their inter-domain linker

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Abbreviations: CD, Circular dichroism; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSQC, heteronuclear single quantum correlation; ICP-AE, inductively coupled plasma-atomic emission; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; LMCT, ligand to metal charge transfer; Mops, 3-morpholinopropanesulfonate; TOF, time of flight.

Abstract

Copper-transporting P-type ATPases, which play important roles in trafficking Cu(I) across membranes for the biogenesis of copper proteins or for copper detoxification, contain a variable number of soluble metal-binding domains at their N-termini. It is increasingly apparent that these play an important role in regulating copper transport in a Cu(I)-responsive manner, but how they do this is unknown. CopA, a Cu(I)-transporter from *Bacillus subtilis*, contains two N-terminal soluble domains that are closely packed, with inter-domain interactions at two principal regions. Here, we sought to determine the extent to which the domains interact in the absence of their inter-domain covalent linker, and how their Cu(I)-binding properties are affected. Studies of a 1:1 mixture of separate CopAa and CopAb domains showed that the domains do not form a stable complex, with only indirect evidence of a weak interaction between them. Their Cu(I)-binding behaviour was distinct from that of the two domain protein and consistent with a lack of interaction between the domains. Cu(I)-mediated protein association was observed, but this occurred only between domains of the same type. Thus, the inter-domain covalent link between CopAa and CopAb is essential for inter-domain interactions and for Cu(I)-binding behaviour.
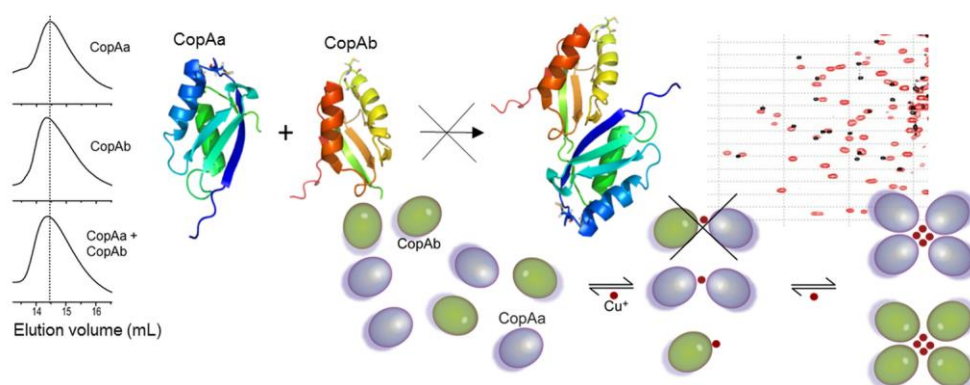
Highlights

- Metal-binding domains of CopA do not form a stable complex without a covalent link

- The domains weakly interact, affecting stability and Cu(I)-binding behaviour

- Cu(I)-dependent domain association occurs only between domains of the same type

Key Words

Copper trafficking, metallochaperones, homeostasis, metal transport.

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ToC graphic

Introduction

Copper-transporting P-type ATPases, which belong to the P_{IB-1} subgroup of the P-type ATPase superfamily, have been identified in a wide variety of organisms, in which they function in moving Cu(I) across membranes, either for detoxification or for the biogenesis of a copper-containing protein [1, 2]. Like all P-type ATPase transporters, they are integral membrane proteins with soluble phosphorylation, actuator and nucleotide-binding domains. The membrane-spanning region of these transporters, which consists of six to eight transmembrane helices, includes a CPX (cysteine-proline-cysteine/histidine/serine) motif that plays a key role in determining the specificity for Cu(I).

Also unique to the P_{IB-1} subgroup is a variable number of usually N-terminal metal-binding domains. In most cases, these have a ferredoxin-like βαββαβ fold and contain a conserved MXCXXC copper-binding motif [3]. Bacterial transporters feature either one or two metal-binding domains, while in eukaryotic transporters they number between two (e.g., in yeast) and six (e.g., in human) [4, 5]. Furthermore, the nature of linking sequences between the domains is variable. In bacteria, transporters with two domains normally have a very short linker (a few residues), while in eukaryotes the linker is short between the 5th and 6th domains, but between other domains it varies between 10 – 90 residues [4].

The functions of the domains have been the subject of considerable debate. While some reports indicated that they are involved directly in the mechanism of Cu(I) transport [6], others revealed that they are not essential for Cu(I) transport [7-9]. Consistent with this, the X-ray structure of the Cu(I) transporter from *Legionella pneumophila* [10] and a cryo-EM based model of CopA from *Archaeoglobus fulgidus* [11] showed that the soluble domains are too far away from the proposed Cu(I)-entry site to be directly involved in transport. However, proximity to the actuator and nucleotide-binding domains, along with protein-protein interaction studies, led to the proposal that the metal-binding domain(s) fulfill a regulatory function through interactions with these domains [8, 10-12].

Human copper-transporting P-type ATPases ATP7A (Menkes protein) and ATP7B (Wilson protein) each possess six N-terminal soluble domains [13]. The function of these domains is more complex than in their bacterial or yeast counterparts because they play an important role in trafficking

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the transporter to different cellular compartments in response to variance in copper levels [14]. While each soluble domain of ATP7A can bind Cu(I) [3, 15], a protein containing all six domains preferentially converted to a more stable form with 4:1 stoichiometry [16], suggesting that Cu(I)-induced changes in tertiary structure result from cooperative interactions of the six N-terminal domains of ATP7A. On the other hand, NMR studies showed that the first and fourth domain formed a metal-mediated adduct with Hah1, the cognate copper chaperone, with Cu(I) then transferring to domains 5 or 6 [15, 17]. *In vivo* studies showed that either the fifth or sixth domain alone was sufficient for trafficking to the plasma membrane/copper transport [18]. A similar picture has emerged for ATP7B, in which all domains are capable of binding Cu(I) [19] but only domains 5 and 6 are important for Cu(I)-dependent cellular localisation [20], and domains 1, 2 and 4 appear to interact preferentially with Hah1 [21].

The Cu(I)-transporting P-type ATPase from *Bacillus subtilis*, CopA, contains two soluble N-terminal domains with a two amino acid residue linker [22, 23]. Both domains are able to bind Cu(I) and to interact with the chaperone CopZ [24]. The structure of the two domain protein CopAab revealed that it is organised in two closely packed ferredoxin-like domains, linked through only two residues, Val72 and Thr73, and spatially oriented such that the two Cu(I)-binding sites are far apart, see Fig. 1 [22, 23]. There are two interacting regions between the two domains, one involving residues 11–12 and 72 with residues 119–122 and the other involving residues 9–10, 57, and 61 with residues 103–104. The protein undergoes Cu(I)-mediated dimerisation above 1 Cu(I) per protein, leading to a luminescent Cu(I) cluster [23, 25]. Each soluble domain has also been studied in isolation, revealing different stabilities and, although both bind Cu(I), they do so in different ways [26, 27], suggesting that they are functionally distinct.

In order to determine the importance of the covalent linkage between the two domains of CopAab, we employed spectroscopic and bioanalytical methods to study 1:1 mixtures of the separate CopAa and CopAb domains. The data reveal that, in the absence of the covalent link, the domains do not form a stable complex, with only indirect evidence of a weak interaction. Although Cu(I)-mediated domain association was observed, this occurred only between domains of the same type, consistent with a lack of inter-domain interaction. The physiological consequences of this are discussed.
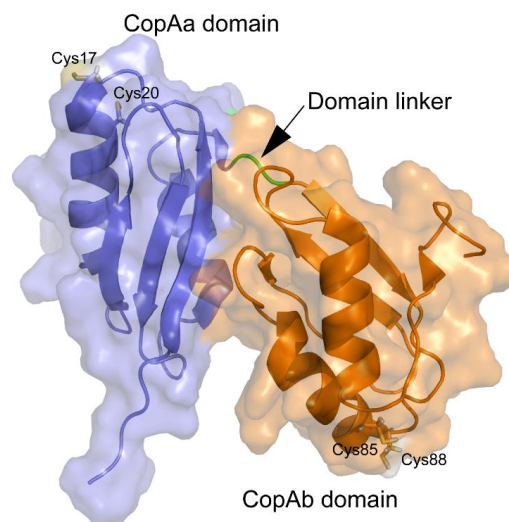
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Figure 1. The N-terminal metal binding domains of CopA. The structural image was generated using PyMol (www.pymol.org) [28] with PDB file:2RML [23]. The two domain protein is called CopAab, while the individual domains are CopAa and CopAb, as indicated in blue and orange, respectively. The two residue linker connecting the domains is shown in green. Cys residues at the Cu(I)-binding motifs of each domain are shown.

1. Material and Methods

2.1 *Purification of CopAa and CopAb and additions of Cu(I) ions*

CopAa and CopAb containing, respectively, residues 1 – 72 and 73 – 147 of *B. subtilis* CopA were purified as previously described [26, 27] using an Äkta Prime Plus (GE Healthcare). CopAa is known to be less stable than CopAb [26, 27, 29]. Unfolding appears to be concentration dependent because there was no evidence for it occurring prior to the dilution of CopAa from concentrated stock solution to generate working solutions for experiments. During experiments, precipitation did not occur (as evidenced by the lack of scattering measured by UV-visible absorbance spectroscopy). Prior to the addition of metal ions, the protein solutions were reduced using 5 mM DTT and excess DTT was subsequently removed using a desalting column (PD-10, GE Healthcare). The oxidation state of the cysteines following removal of DTT was assessed by reaction with Ellman's Reagent (5'5-dithio-bis (2-nitrobenzoic acid), DTNB), which confirmed the presence of ~2 reactive thiols per protein molecule.

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Anaerobic additions of Cu(I) were made using a microsyringe (Hamilton) as previously described [23, 26, 27]. Titration experiments were conducted using a single sample cuvette; after each metal ion addition, samples were incubated for 2 min before spectra were recorded. Data were corrected for dilution effects.

2.2 *Absorbance and CD spectroscopies*

UV-visible absorbance spectra were recorded on a Perkin-Elmer λ35 or JASCO V550 spectrophotometer. CD spectra in the far-UV range (190-250 nm) were recorded using a Jasco J-810 spectropolarimeter interfaced to a PC, with a slit width of 2 nm and scan speed of 100 nm/min. CD intensity is expressed as molar ellipticity ($[\theta]$) in units of deg cm^2 dmol^{-1}. Spectra were corrected for intensity due to the buffer. Fluorescence emission spectra were recorded using a Perkin-Elmer LS55 spectrophotometer at 25 ℃ with excitation at 276 nm and excitation and emission slit widths of 9 nm. For the measurement of emission in the region 500 – 700 nm, slit widths were set to 10 nm (with excitation at 295 nm) and a 390 nm cut-off band pass filter was employed. Scan speed was 200 nm/min for all fluorescence measurements.

2.3 *NMR spectroscopy*

NMR spectra were acquired using a Bruker Avance III 800 MHZ spectrometer equipped with a triple resonance, pulsed field gradient probe, operating at frequencies of 800.23 MHz (^{1}H) and 81.09 MHz (^{15}N), using pulse sequences incorporated into the Bruker Topspin 2.1 software. Reduced apo-^{15}N-CopAa and apo-CopAb (300 μM of each in 100 mM phosphate, pH 7.0, 10% D$_2$O) were loaded into an NMR tube and 2D ^{1}H-^{15}N HSQC spectra were recorded at 298 K and processed using NMRPipe [30]. The ^{1}H carrier frequency was positioned at the resonance of the water during the experiments, and the ^{15}N carrier frequency was at 115 ppm. Prior to Fourier transformation, a cosine-bell window function was applied to each dimension for apodization. The indirect dimensions were first linear-predicted to double the number of data points, and then zero-filled to round up the number of data points to the nearest power of 2.

2.4 *Analytical gel filtration*

Samples were prepared under anaerobic conditions containing 100 µM of each CopAa and CopAb in 100 mM MOPS, 100 mM NaCl, pH 7.5, at 0.5, 1.0 and 2.0 Cu(I) per domain. Each 500 µl sample was loaded onto a Superdex 75 10/300 GL column (GE Healthcare), with a total column volume (Vt) of 24 ml and a void volume (V_0) of 7.6 ml, as determined using blue dextran. The column was equilibrated and operated using thoroughly deoxygenated buffer at a flow rate of 0.8 ml min^{-1}. Elution fractions were collected every 0.5 mL and analysed for copper and protein.

2.5 *Copper analysis*

For analysis of the elution fractions from the G75 column, an ICP-AE (inductively coupled plasma atomic emission) spectrometer (Varian Vista) was used, calibrated with ICP-AE grade Cu standards (Fisher) diluted to appropriate concentrations. After overnight incubation in 50% (v/v) nitric acid (Primar Plus, Fisher) at 60 °C, elution buffer (as baseline) and protein samples were diluted to appropriate concentrations in a final volume of 10 ml using HiPerSolv water (VWR) for a final concentration of 4% (v/v) nitric acid. Spectral line at 324.754 nm was used to determine copper concentrations. Data analysis was performed using ICP Expert version 4.1.

2.6 *Mass spectrometry*

HPLC-MS experiments were performed using an UltiMate 3000 HPLC system (Dionex, Sunnvale, CA, USA), and a Bruker micrOTOF-QIII electrospray ionization (ESI) time-of-flight (TOF) mass spectrometer (Bruker Daltonics, Coventry, UK), in positive ion mode. Protein samples were diluted 50-fold into 2% (v/v) acetonitrile and a 1 µl injection volume was applied to a ProSwift® reversed phase RP-1S column (4.6 x 50 mm; Dionex) at 25 °C. Gradient elution was performed at a flow rate of 200 µl/min using solvents A (0.1% (v/v) formic acid) and B (acetonitrile, 0.1% (v/v) formic acid), with the following chromatographic method: isocratic wash (2% B, 0–1 min), linear gradient from 2–100% B (1–3 min), followed by an isocratic wash (100% B, 3–7 min) and column re-equilibration (2% B, 7–11 min). Mass spectra were recorded in the range 500 – 3400 m/z, using Bruker oTOF Control software with parameters as follows: dry gas flow 8 L/min, nebuliser gas pressure 1.8 bar, dry gas 240 °C,

capillary voltage 4500 V, offset 500 V, collision RF 650 Vpp. The ESI-TOF was calibrated in the m/z range 300 - 2200 using ESI-L Low Concentration Tuning Mix (Agilent Technologies, San Diego, CA). Processing and analysis of MS experimental data was carried out using Compass DataAnalysis version 4.1 (Bruker Daltonik, Bremen, Germany). The spectra were deconvoluted using the ESI Compass version 1.3 Maximum Entropy deconvolution algorithm over a mass range of 7000 – 20000 Da. Exact masses were determined from peak centroids, with minimal smoothing applied. CopAa domain (7692 Da) was readily distinguished from the larger CopAb domain (8258 Da).

2.   Results

3.1   *NMR studies reveal no significant interaction between CopAa and CopAb.*

The ^{1}H^{15}N HSQC NMR spectrum of ^{15}N-labelled apo-CopAa was recorded and overlaid with that of ^{15}N-labelled CopAab, Fig. 2A. Most of the resonances of CopAa overlay with the equivalent peaks of the two domain protein [23]. However, some resonances, and in particular those due to residues located within the secondary structure elements β1 (Ile8, Ala9, Met10, Gln11, Val12), α1 (Lys26, Gly27, Leu28), β2 (Val39, Asn40), and the end of β4 (Val71, Val72) are shifted. Residues within the β1 and β4 elements are affected by inter-domain contacts, as they are close to or at the surface interacting with CopAb residues in the two domain protein (Fig. 2B). Why residues in the α1 and β2 elements, which are remote from the domain interface, should be affected is not so clear but such long-range chemical shift perturbations are not uncommon, and often reflect a variation in hydrogen-bond strength for the relevant peptide NH groups [31].

To investigate physical interactions between CopAa and CopAb in the experimental mixture of two proteins, ^{15}N-labelled apo-CopAa and non-labelled apo-CopAb were mixed in 1:1 ratio. The resulting ^{1}H^{15}N-HSQC spectrum was essentially identical to that of apo-CopAa alone (Fig. 2C shows the two spectra shifted by 0.03 ppm to assist visualisation), indicating that no significant interaction between the two domains in the absence of a covalent link between them is detected by NMR.
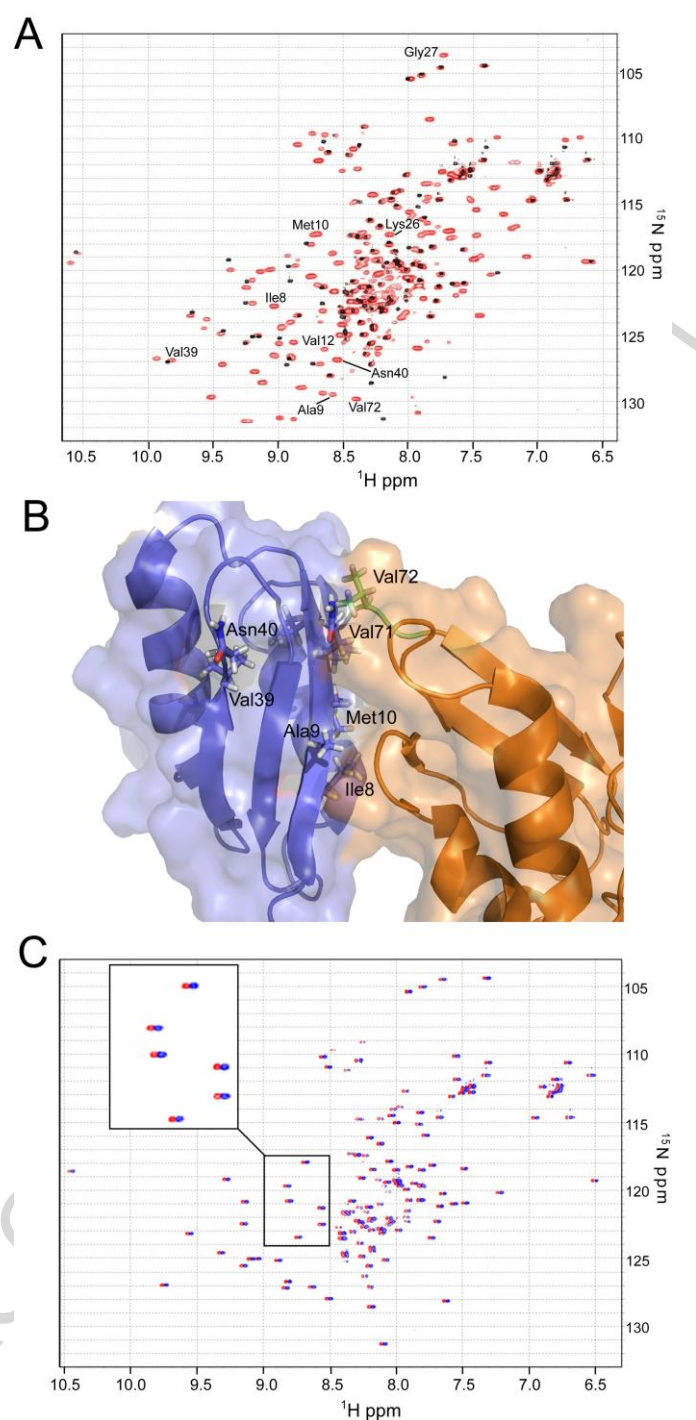
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Figure 2. Investigation of CopAa conformational changes due to CopAb. A) $^1H^{15}N$-HSQC NMR overlaid spectra at 800 MHz and 298 K of apo-CopAa (200 µM, in black) and apo-CopAab (160 µM, in red) in 100 mM phosphate buffer, pH 7.0 with 10% D_2O. **B**) A cartoon/surface diagram of CopAab derived from the NMR structure [23] showing residues for which resonances are shifted in the NMR spectrum of isolated CopAa compared to those of the same domain in the spectrum of CopAab. Visualisation was achieved using PyMol [28] with PDB file:2RML. **C**) $^1H^{15}N$-HSQC NMR spectrum

of a mixture of ^{15}N labelled apo-CopAa and non-labelled apo-CopAb (300 µM in each, in red) overlaid with spectrum of ^{15}N-labelled apo-CopAa (200 µM, in blue). Overlaid blue spectrum was shifted by +0.03 ppm relative to red to facilitate visualisation of both spectra. A region of the spectrum is shown in magnified form.

3.2   The conformational stability of CopAa is increased in the presence of CopAb.

Previous studies of CopAa showed that it is much less stable than CopAb in the absence of Cu(I) [26, 27] and that it undergoes Cu(I)-induced unfolding, particularly at levels >1 Cu(I) per CopAa [26]. Far UV CD studies of apo-CopAa confirmed that it is only marginally stable. Fig. 3A shows spectra recorded immediately following reduction and removal of excess DTT, and following overnight incubation. Clearly significant secondary structure loss occurred during this period. A 1:1 mixture of apo-CopAa and apo-CopAb gave a spectrum, Fig. 3B, indicative of both β-sheet and α-helix content, similar to apo-CopAab [23] except that the positive peak is slightly shifted to <195 nm and the negative peak observed for apo-CopAab at 222 nm is not as well defined in the mixture of separate domains (inset, Fig. 3A). Re-measurement of the spectrum (Fig. 3B) after overnight incubation revealed that only a small amount of secondary structure was lost, indicating that the presence of apo-CopAb resulted in a stabilisation of the CopAa fold.
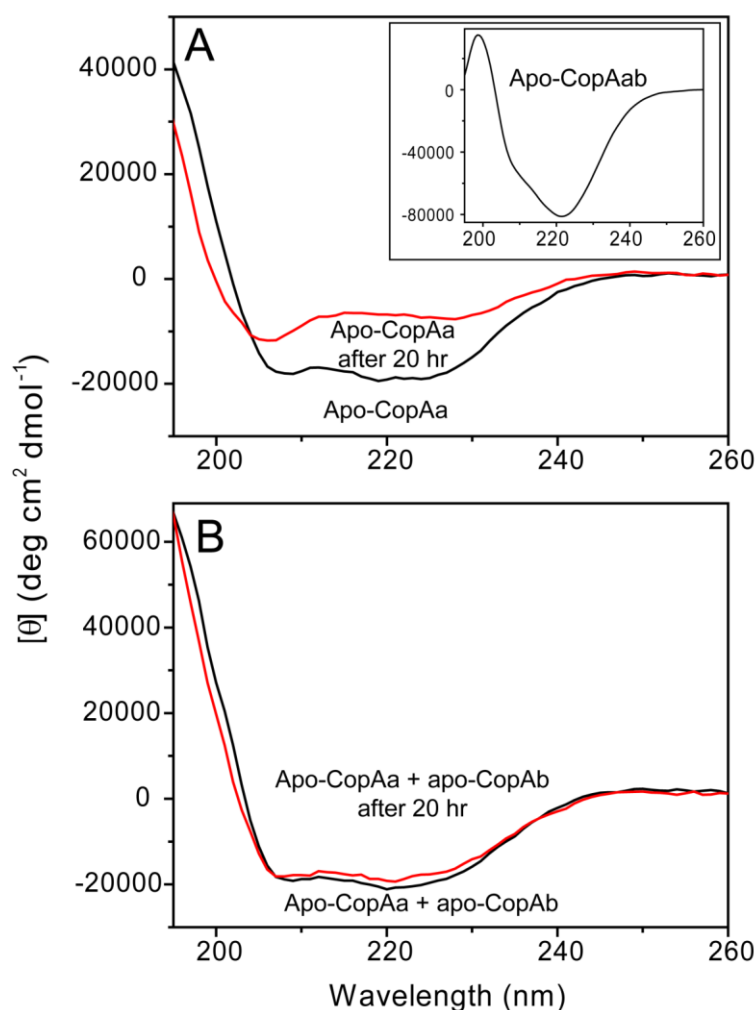
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Figure 3. Far UV CD characterisation of CopAa conformational stability. A) Far UV CD spectra of pre-reduced apo-CopAa (15 µM) in 50 mM phosphate buffer, pH 7.5, immediately after removal of reductant and after 20 hr incubation at 16 °C, as indicated. **B**) As in A, but with the addition of apo-CopAb (15 µM in each). The spectrum of CopAab (21 µM) in 100 mM phosphate buffer, pH 7.0 is shown for comparison (previously published in ref [23]).

3.3   Spectroscopic studies of Cu(I)-binding in a CopAa/CopAb mixture reveals largely

independent binding.

Additions of Cu(I) were made to a 1:1 mixture of CopAa and CopAb and UV-visible absorption spectra recorded after each addition, see Fig. 4A. Plots of $\Delta A_{265\ nm}$ and $\Delta A_{295\ nm}$ as a function of Cu(I) per domain (i.e., CopAa + CopAb) are shown inset. Note that it is also useful to express Cu(I) in absolute concentration terms, see Fig. S1A. The data indicate that binding of Cu(I) to the CopAa/CopAb mixture

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is complex and multi-phasic. Three phases are apparent in the approximate ranges 0 – 0.7, 0.7 – 1.5 and > 1.5 Cu(I)/domain.
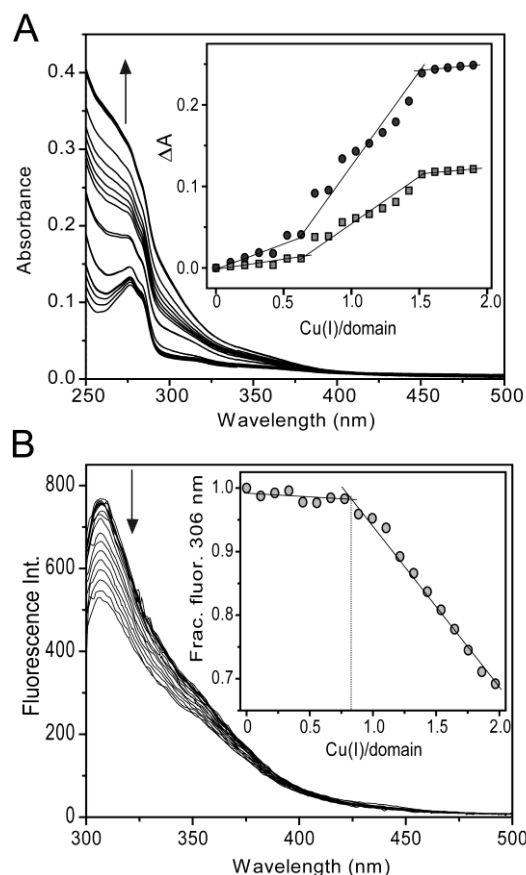

Figure 4. Absorbance and fluorescence characterisation of Cu(I)-binding to CopAa and CopAb.
A) UV-visible absorbance spectra of a 1:1 mixture of CopAa and CopAb following the addition of 0-70 µM Cu(I). Proteins (15 µM in both CopAa and CopAb) were in 100 mM MOPS, pH 7.5, path length 1 cm. Inset, Plots of absorbance changes at 265 nm (circles) and 292 nm (squares) as a function of Cu(I) per domain. **B**) Fluorescence spectra of a CopAa/CopAb mixture following the addition of 0-20 µM Cu(I). Proteins (5 µM in both) were in 100 mM MOPS, pH 7.5. Excitation and emission slit widths were 9 nm. Inset, Plot of fractional fluorescence at 306 nm as a function of Cu(I) /domain.

The overall pattern of absorbance changes observed here are similar to those previously reported for Cu(I)-binding to CopAab [23, 25], particularly in the first part of the titration. Data for CopAab were previously reported in terms of Cu(I) per CopAab protein and so to convert these values to 'per domain' requires division by two, see Fig. S2A. CopAab undergoes dimerisation at a Cu(I)-loading of > 0.5 Cu(I)/domain (or 1.0 Cu(I)/protein), and this is reflected in the UV-visible titration data for CopAab, with different binding phases below and above 0.5 Cu(I)/domain. In addition, there is a

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further break at 1 Cu(I)/domain (or 2 Cu(I)/protein). Thus, while the titration data for CopAa/CopAb are similar to those for CopAab up to ~1 Cu(I)/domain, above this level they are very different.

Cu(I)-binding to the isolated domains CopAb and CopAa as separate solutions have previously been investigated in detail [26, 27]. These studies revealed distinctly different behaviours. CopAa gave four distinct phases with break points at 0.5, 1.0, and 1.5 Cu(I)/CopAa [26], while CopAb gave three distinct phases with break points at 1.0 and 2.0 Cu(I)/CopAb [27]. The initial binding of Cu(I) to the two domains was shown to occur with similarly high affinity, and so addition of Cu(I) to a mixture of the two domains would be expected to result in binding to both. Given that a break in the titration $\Delta A_{265 nm}$ plot would be expected at 0.5 Cu(I)/domain for CopAa alone and at 1 Cu(I)/domain for CopAb alone, independent binding of Cu(I) to a mixture of CopAa and CopAb would be expected to give a phase intersection at ~0.75 Cu(I) per domain, i.e., summation of the binding data for the individual domains would give an intersection at ~0.75 Cu(I) per domain, close to what was observed here. Behaviour beyond initial binding is more difficult to predict because the relative affinities of higher order Cu(I)-binding to CopAa and CopAb are not known. However, if it were assumed that these were similar, a further break in the titration would be expected at ~1.5 Cu(I)/domain. These stoichiometric ratios match the experimental data well, consistent with Cu(I)-binding to the two domains occurring independently. However, neither the Cu(I) titration data for CopAa nor CopAb alone features a plateau of the type observed for the CopAa/CopAb mixture at >1.5 Cu(I)/domain, suggesting that some interaction between domains occurs.

CopAa and CopAb each contain two fluorescent tyrosine residues, giving rise to an emission band at 306 nm [23, 25]. Addition of Cu(I) ions to a CopAa and CopAb mixture resulted in a quenching of fluorescence intensity, Fig. 4B. A plot of fractional fluorescence against Cu(I)/domain (inset) shows that initial additions of Cu(I) did not cause a significant change in fluorescence intensity. However, quenching occurred above ~ 0.8 Cu(I)/domain. Again, a similar pattern of behaviour was previously observed for CopAab (Fig. S2B), with quenching beginning at >0.5 Cu(I)/domain (>1.0 Cu(I)/CopAab). The isolated domains exhibited multi-phasic binding behaviour similar to that described above for absorbance, and so, as above, if independent high affinity binding occurred to both domains, quenching would be observed at 0.75 Cu(I)/domain (a summation of the binding behaviour of the individual

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domains). Thus, UV-visible and fluorescence data indicate that Cu(I)-binding to the CopAa/CopAb mixture occurs largely independently to the two domains.

An equivalent titration to those above for the CopAa/CopAb mixture was also monitored by luminescence spectroscopy, see Fig. 5A [23, 25]. Addition of Cu(I) resulted in the formation of one or more weakly luminescent species with emission centred around 600 nm. The intensity change at 610 nm was plotted as a function of Cu(I) per domain, Fig. 5B (and versus Cu(I) concentration in Fig. S1C). This showed an initial shallow increase upon addition of 0 – 0.25 Cu(I)/domain, followed by a more substantial increase up to 0.5 Cu(I)/domain, which then remained stable at higher levels of Cu(I), up to 2.5/domain. This behaviour is very different from that observed for CopAab, which below a level of ~0.5 Cu(I)/domain (~1 Cu(I)/CopAab) does not give rise to any luminescence intensity (Fig. S2C) [25]. Above this level, however, a highly luminescent species is formed with maximum intensity (>10-fold greater than observed here) at ~1 Cu(I)/domain (~2 Cu(I)/CopAab). Clearly, the mixture of CopAa and CopAb does not mimic the properties of CopAab.
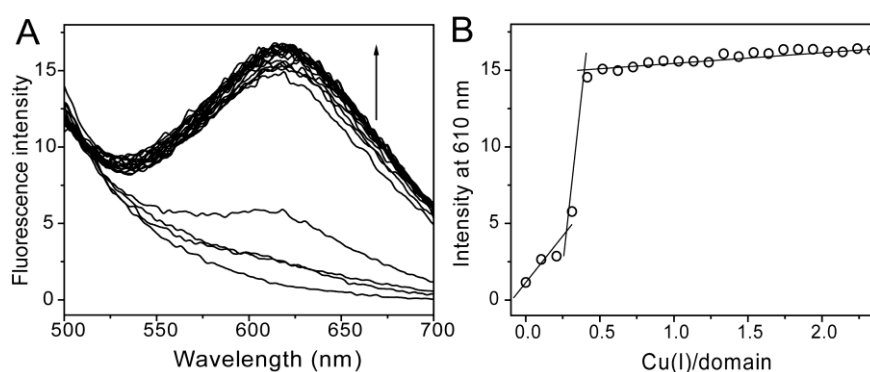

Figure 5. Luminescence characterisation of Cu(I)-binding to CopAa and CopAb. A) Luminescence spectra of a 1:1 mixture of CopAa and CopAb (5 µM of each protein) in 100 mM MOPS, pH 7.5 following the addition of 0- 20 µM Cu(I). **B**) Plot of change in luminescence at 610 nm as a function of Cu(I) per domain.

Previous studies of the isolated domains showed luminescence increases for CopAa from ~0.25 – 1 Cu(I)/CopAa [26], while for CopAb, luminescence intensity did not appear until ~ 1 Cu(I)/CopAb [27]. In both cases, only low intensity luminescence (similar to that observed here for the mixture) was observed. Thus, the luminescence titration behaviour cannot be interpreted as the sum of the individual

behaviour of the separate CopAa and CopAb domains. Therefore, while the UV-visible and fluorescence data point largely to independent binding of Cu(I) to CopAa and CopAb, the luminescence data indicate that the two domains must interact to some extent in solution, resulting in perturbed Cu(I)-binding behaviour.

3.4   Cu(I)-mediated domain association occurs only between domains of the same type.

Analytical gel filtration was used to gain insight into the species present when Cu(I) is added to a mixture of CopAa and CopAb. An equimolar mixture of apo-CopAa and apo-CopAb resulted in a major peak at the same position as that observed for the two individual domains (Fig. S4). These have been shown previously to both be monomeric [26, 27]. Thus, the data are consistent with NMR data in that they indicate that the two domains do not associate to form a stable complex in solution. Some low intensity bands were observed at lower elution volumes, indicating that a small proportion of protein was present as larger species (Fig. S4).

Addition of 0.5 Cu(I)/domain resulted in a chromatogram (Fig. 6A) containing a major peak at an elution volume of ~14 ml and a shoulder at elution volume ~13 ml. This indicates that the sample is in the form of monomers together with a higher molecular mass species (eluting earlier) resulting from Cu(I)-induced monomer association. This could be due to homo- or hetero-species containing CopAa and/or CopAb. To investigate this, the protein and copper distribution across the gel filtration elution profile were analysed using ICP-AE (for copper) and LC-MS (for proteins), see Fig. 6A. These revealed that the main (monomer) peak is due to both CopAa and CopAb and contained the majority of copper. The shoulder at ~13 ml is due primarily to CopAa, consistent with this being a higher association state form of CopAa. Only low amounts of copper were detected in these fractions but it is likely that these result from copper-mediated association. Previous studies of CopAa showed that addition of Cu(I) initially resulted in formation of CopAa tetramers, but the elution volume for the tetramer species was 11 ml [26], suggesting that the species formed here is significantly smaller, most likely a dimer [26]. This suggests that the presence of CopAb inhibits Cu(I)-induced CopAa tetramer formation.
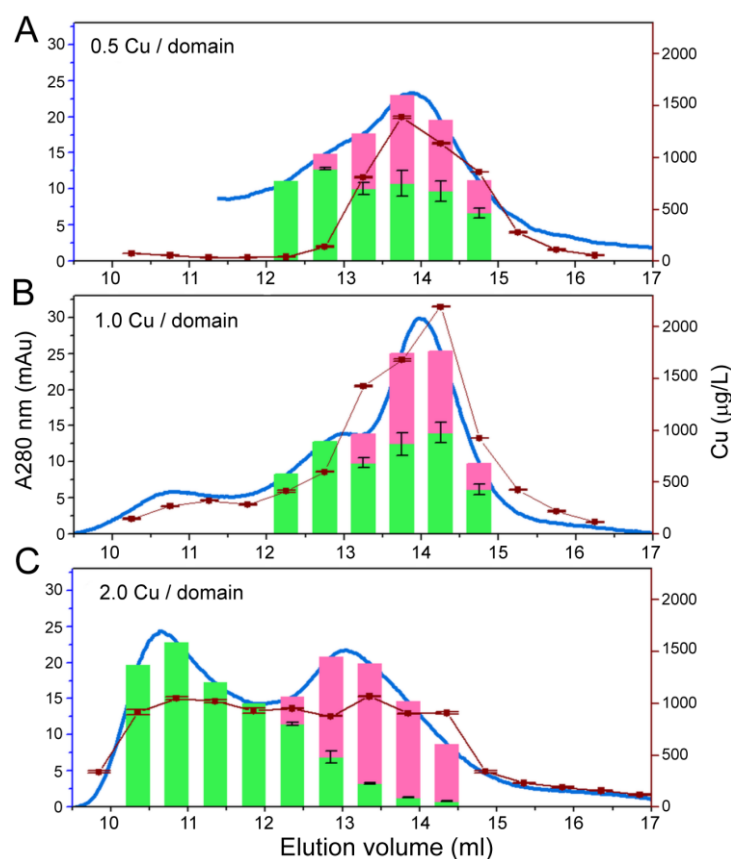
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Figure 6. Copper distribution between CopAa and CopAb. Cu(I) was added to samples containing CopAa and CopAb (100 μM in each) in 100 mM MOPS, 100 mM NaCl, pH 7.5, to give: **A)** 0.5, **B)** 1.0, and **C)** 2.0 Cu(I) per domain. Each sample was subjected to gel filtration (blue trace, chromatogram at A$_{280\,nm}$, left ordinate). Elution fractions were analysed by ICP-AE for Cu(I) (red trace, µg/L, right ordinate), and by LC-MS for CopAa (green bars) and CopAb (magenta bars). Relative protein levels are plotted, with the height of the combined bar plot set to match that of the elution absorbance trace. The plot illustrates the changes in association state of CopAa and CopAb with increasing copper level, and binding of copper by each of the observed forms.

At 1 Cu(I)/domain, the chromatogram (Fig. 6B) again showed a major peak at ~14 ml, a shoulder at ~13 ml and a more distinct shoulder at ~10.7 ml. Protein and copper analyses revealed that the main (monomer) peak is due to both CopAa and CopAb and again contained the majority of the copper. The ~13 ml shoulder was due only to CopAa and with significant amounts of copper. The lower elution volume peak was also associated with copper but protein was not detected by LC-MS in these fractions.

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The effects of Cu(I) on CopAa in a mixture of CopAa (^{15}N- labelled) and CopAb (unlabelled) were also investigated by NMR. Addition of 1 Cu(I)/domain resulted in a ^{1}H^{15}N-HSQC spectrum in which only resonances in the 6.7-8.5/109-128 ppm (^{1}H/^{15}N) region were observed (Fig. S3). As observed previously for 0.5 Cu(I)/CopAa [26], the data indicate that CopAa undergoes association upon binding Cu(I), resulting in the loss of a well dispersed NMR spectrum.

At 2 Cu(I)/domain, two main peaks were observed in the chromatogram (Fig. 6C), at elution volumes of ~13 ml and ~10.7 ml. Protein and copper analyses revealed that the broad peak at ~13 ml is due mostly due to CopAb, and likely represents a copper bound dimeric form [27]. The peak at ~10.7 ml results only from CopAa and corresponds to a copper-bound tetrameric form [26]. The CopAa profile is asymmetric with a tail that extends into the CopAb peak, suggesting that a dimeric form of CopAa is also present but at lower concentration than the tetramer.

3. Discussion

The two N-terminal domains of CopA closely pack to form a single rigid molecule with a two residue (Val72 and Thr73) linker between domains [22, 23]. There are two interacting regions between the two domains, the first involving residues 11/12 and 72 (CopAa) and residues 119–122 (CopAb), and the second residues 9/10, 57, and 61 (CopAa) and residues 103–104 (CopAb). These interactions are in part due to an H-bond network at the inter-domain region, involving the pairs: Hε2 Gln11/Oε2 Glu122; backbone NH Gln11/Nδ2 Asn119; and Hδ2 Asn104/Oε1 Gln61. Hydrophobic interactions are not a major part of the inter-domain contacts, being limited to the interactions between Met10 and Ala103 and between Val72 and the side chain of Lys121 [22].

Here, HSQC NMR data for ^{15}N-labelled CopAa alone was compared with that of ^{15}N-labelled CopAab (Fig. 2A). Most of the resonances of CopAa were found to overlay with the equivalent peaks of the two domain protein, with exceptions for residues principally at or close to the surface interacting with CopAb residues in the two domain protein (Fig. 2B). The HSQC spectrum of a mixture of ^{15}N-labelled CopAa with unlabelled CopAb was then compared with that of ^{15}N-labelled CopAa alone. There were no differences between them (Fig. 2C), demonstrating directly that, under the conditions employed here, there was no interaction between the isolated domains. This also provided an indirect

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comparison between the CopAa/CopAb mix and CopAab. Had there been evidence of an interaction, then it would have been important to more carefully (directly) compare the CopAa/CopAb mix spectrum with that of CopAab, to determine if all of the CopAa residue resonances aligned with those for the CopAa domain in the CopAab spectrum. The lack of interaction between isolated domains was also apparent from gel filtration studies of the apo-proteins (Fig.S4). Thus, despite the inter-domain interactions observed in the two domain protein CopAab, in the absence of the covalent link between them, the domains do not form a stable complex.

Studies of interactions between the N-terminal metal binding domains of the human copper transporters ATP7A and ATP7B, which contain six such domains, have revealed that, while domains 5 and 6 form a compact structure, overall, the six domains do not [15, 21, 32]. Instead, they undergo transient interactions with each other that are likely to be important for both copper transfer and for regulation of transfer activity [33]. In particular, domains 1-3 of ATP7A were shown to interact with each other in a highly dynamic, transient fashion [34], and very recent NMR studies of interactions between the separately purified domains showed a clear interaction between domains 1 and 3, and some evidence for weaker interactions between domains 1 and 2 and 2 and 3 [35].

Where highly stable inter-protein interactions are found, interactions are commonly maintained in the absence of a covalent linkage between the different parts of the proteins, as illustrated by the classic studies of single domain proteins such as RNase A and barnase [36, 37]. Thus, the data presented here for the N-terminal domains of *B. subtilis* CopA are unusual in that they show that a stable complex does not form between the isolated domains even though they are known to interact in the two domain protein CopAab [23]. This may well be consistent with the emerging picture of dynamic, transient interactions between the N-terminal domains of the human ATP7A and ATP7B proteins; it seems that in the absence of the covalent link, the domain-domain interactions are not sufficiently strong to maintain a complex. Studies of interactions between the N-terminal domains of other prokaryotic copper transporting P-type ATPases would be interesting to determine if this behaviour is typical.

Studies of the Cu(I)-binding properties of a 1:1 mixture of CopAa and CopAb by UV-visible absorbance and fluorescence (Fig. 4) were consistent with previous spectroscopic studies that showed that the two domains initially bind Cu(I) ions with similarly high affinity [26, 27], and could be

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reasonably interpreted as resulting from entirely independent binding of Cu(I) to the two domains, consistent with the conclusion that the domains do not interact.

While it is clear that the two domains do not form a stable complex, the data did provide indirect evidence of a weak interaction. Far UV CD spectra (Fig. 3) demonstrated a positive effect on the conformational stability of CopAa in the presence of CopAb, and luminescence data (Fig. 5) revealed behaviour that could only result from an interaction between the domains that influences Cu(I)-binding to one or both. Furthermore, gel filtration revealed that the Cu(I)-dependent association state properties of the CopAa domain were affected by the presence of CopAb. The tetrameric species observed to form immediately upon Cu(I)-binding to CopAa alone [26] was only observed at higher Cu(I) concentrations, and instead a likely dimeric CopAa species was observed at lower Cu(I) levels. Thus, a weak interaction between the domains must occur. Given the concentrations employed in NMR and gel filtration experiments, we estimate that the K_d for complex formation is no lower than the mM level. For future studies, it would be interesting to try to find direct evidence for this weak interaction between the domains. While FRET/EPR or cross-linking are often employed to detect protein-protein interactions, NMR is normally the method of choice when looking for evidence of weak interactions (because it can detect these where other methods cannot) and so the best strategy for this work is unclear at present.

It was previously shown that the short inter-domain linker precludes intra-protein, inter-domain Cu(I)-binding (whereby a Cu(I) ion would bridge the CopAa and CopAb binding motifs), because it results in a spatial orientation of domains that places the two Cu(I)-binding sites far apart [22, 23]. In the absence of the constraints imposed by the linker, inter-domain Cu(I)-binding could occur. Gel filtration/copper and protein analyses reported here (Fig. 6) demonstrate that Cu(I)-mediated hetero-complexes of the two domains do not accumulate, consistent with the lack of a stable interaction between the apo-proteins. The previously reported dimerisation of CopAab that occurs >1 Cu(I) per protein [23, 25] could be due to homo- or hetero-domain interactions. We conclude that CopAab dimerisation very likely occurs via homo-domain interactions, probably initially via CopAa, which dimerises much more readily than CopAb [26, 27].

The function(s) of the N-terminal soluble domains of Cu(I)-transporting P-type ATPases is not entirely clear, though there is growing evidence that they play a key role in regulating the activity of

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the transporter. In the crystal structure of *L. pneumophilia* CopA, the N-terminal soluble domain was not well-defined, but an approximate location was modelled, indicating proximity to the actuator and nucleotide-binding domains [10], consistent with cryo-EM studies [11]. While the N-terminal domains of the human ATP7B transporter do not interact with other cytoplasmic domains [35, 38], the very N-terminal part of the protein (that precedes the first metal binding domain) was recently shown to bind to the nucleotide-binding domain [35], thus accounting for earlier observations of a Cu(I)-dependent interaction between the N-terminal part of the protein and the nucleotide-binding domain [12]. A Cu(I) and ATP-sensitive interaction between the N-terminal soluble and nucleotide-binding domains of *A. fulgidus* CopA has also been reported [39], indicating that regulatory interactions also occur in prokaryote transporters.

Thus, a picture of how regulation of copper transporters could occur is evolving [8, 10, 11, 35]. Under low Cu(I), interactions between protein N-terminal domains and the nucleotide-binding domain would inhibit the conformational changes needed in the latter for efficient Cu(I) transfer. As Cu(I) levels increase, Cu(I)-binding to the soluble domains results in changes to the dynamics of domain-domain interactions that reduce the interaction of the N-terminal part with the nucleotide-binding domain and consequently leads to activation of the transporter. In this context, significant Cu(I)-dependent conformational changes are observed for *B. subtilis* CopAab [23, 25], which are dependent on the linker between the domains, and these may be important for such a regulatory role. Whether dimerisation of the N-terminal domains, observed here to involve homo-domain complexes only, has functional relevance is not clear, though we note recent evidence that ATP7B forms a dimer *in vivo* [40].

In conclusion, in the absence of the covalent linker, the two *B. subtilis* CopAa domains do not form a stable complex and behave largely independently of one another, exhibiting Cu(I)-binding properties that are distinct from those of the linked, two domain protein [23]. Thus, both the extent of interaction between the two N-terminal soluble domains of CopA and their particular response to Cu(I) is dependent on their covalent connection. In the context of a likely regulatory function of the N-terminal domains, the different number of domains, and the variable nature of the linkers between them in transporters from different organisms, is likely to result in a variability in the mechanism of regulation. In future studies, it would be interesting to vary the length of the inter-domain linker between

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CopAa and CopAb. A longer linker could allow the domains to adopt different conformations while maintaining a high local concentration that may be sufficient to promote the wild type interaction between them. Alternatively, the relative strain that might result from the short linker in the wild type protein could be important for their interaction. Such studies would reveal the key factors and shed further light on the functional importance of copper transporter N-terminal domain-domain interactions.

References

[1] S. Lutsenko, J.H. Kaplan, Organization of P-type ATPases: significance of structural diversity, Biochemistry 34 (1995) 15607-15613.

[2] I. Voskoboinik, J. Camakaris, J.F. Mercer, Understanding the mechanism and function of copper P-type ATPases, Adv. Protein Chem. 60 (2002) 123-150.

[3] S. Lutsenko, K. Petrukhin, M.J. Cooper, C.T. Gilliam, J.H. Kaplan, N-terminal domains of human copper-transporting adenosine triphosphatases (the Wilson's and Menkes Disease Proteins) bind copper selectively in vivo and in vitro with stoichiometry of one copper per metal- binding repeat, J. Biol. Chem. 272 (1997) 18939-18944.

[4] F. Arnesano, L. Banci, I. Bertini, S. Ciofi-Baffoni, E. Molteni, D.L. Huffman, T.V. O'Halloran, Metallochaperones and metal-transporting ATPases: A comparative analysis of sequences and structures, Gen. Res. 12 (2002) 255-271.

[5] C. Singleton, N.E. Le Brun, Atx1-like chaperones and their cognate P-type ATPases: copper-binding and transfer, Biometals 20 (2007) 275-289.

[6] I. Morin, S. Gudin, E. Mintz, M. Cuillel, Dissecting the role of the N-terminal metal-binding domains in activating the yeast copper ATPase *in vivo*, FEBS J. 276 (2009) 4483-4495.

[7] B. Fan, G. Grass, C. Rensing, B.P. Rosen, *Escherichia coli* copA N-terminal CysX$_2$Cys motifs are not required for copper resistance or transport, Biochem. Biophys. Res. Commun. 286 (2001) 414-418.

[8] A.K. Mandal, J.M. Arguello, Functional roles of metal binding domains of the *Archaeoglobus fulgidus* Cu$^+$-ATPase CopA, Biochemistry 42 (2003) 11040-11047.

[9] M. Gonzalez-Guerrero, J.M. Arguello, Mechanism of Cu$^+$-transporting ATPases: soluble Cu$^+$ chaperones directly transfer Cu$^+$ to transmembrane transport sites, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5992-5997.

[10] P. Gourdon, X.Y. Liu, T. Skjorringe, J.P. Morth, L.B. Moller, B.P. Pedersen, P. Nissen, Crystal structure of a copper-transporting P$_{IB}$-type ATPase, Nature 475 (2011) 59-64.

[11] C.C. Wu, W.J. Rice, D.L. Stokes, Structure of a copper pump suggests a regulatory role for its metal-binding domain, Structure 16 (2008) 976-985.

[12] R. Tsivkovskii, B.C. MacArthur, S. Lutsenko, The Lys1010-Lys1325 fragment of the Wilson's disease protein binds nucleotides and interacts with the N-terminal domain of this protein in a copper-dependent manner, J. Biol. Chem. 276 (2001) 2234-2242.

[13] S. Lutsenko, M.J. Petris, Function and regulation of the mammalian copper-transporting ATPases: insights from biochemical and cell biological approaches, J. Membr. Biol. 191 (2003) 1-12.

[14] S. Lutsenko, N.L. Barnes, M.Y. Bartee, O.Y. Dmitriev, Function and regulation of human copper-transporting ATPases, Physiol. Rev. 87 (2007) 1011-1046.

[15] L. Banci, I. Bertini, F. Cantini, N. Della-Malva, M. Migliardi, A. Rosato, The different intermolecular interactions of the soluble copper-binding domains of the Menkes protein, ATP7A, J. Biol. Chem. 282 (2007) 23140-23146.

[16] P.A. Cobine, G.N. George, D.J. Winzor, M.D. Harrison, S. Mogahaddas, C.T. Dameron, Stoichiometry of complex formation between copper(I) and the N-terminal domain of the Menkes protein, Biochemistry 39 (2000) 6857-6863.

[17] D. Achila, L. Banci, I. Bertini, J. Bunce, S. Ciofi-Baffoni, D.L. Huffman, Structure of human Wilson protein domains 5 and 6 and their interplay with domain 4 and the copper chaperone HAH1 in copper uptake, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 5729-34.

[18] D. Strausak, S. La Fontaine, J. Hill, S.D. Firth, P.J. Lockhart, J.F.B. Mercer, The role of GMXCXXC metal binding sites in the copper-induced redistribution of the menkes protein, J. Biol. Chem. 274 (1999) 11170-11177.

[19] L.A. Yatsunyk, A.C. Rosenzweig, Cu(I) binding and transfer by the N terminus of the Wilson disease protein, J. Biol. Chem. 282 (2007) 8622-8631.

[20] M.A. Cater, J. Forbes, S. La Fontaine, D. Cox, J.F.B. Mercer, Intracellular trafficking of the human Wilson protein: The role of the six N-terminal metal-binding sites, Biochem. J. 380 (2004) 805-813.

[21] L. Banci, I. Bertini, F. Cantini, C. Massagni, M. Migliardi, A. Rosato, An NMR study of the interaction of the N-terminal cytoplasmic tail of the Wilson disease protein with copper(I)-HAH1, J. Biol. Chem. 284 (2009) 9354-9360.

[22] L. Banci, I. Bertini, S. Ciofi-Baffoni, L. Gonnelli, X.-C. Su, Structural basis for the function of the N-terminal domain of the ATPase CopA from *Bacillus subtilis*, J. Biol. Chem. 278 (2003) 50506-50513.

[23] C. Singleton, L. Banci, S. Ciofi-Baffoni, L. Tenori, M.A. Kihlkenl, R. Boetzel, N.E. Le Brun, Structure and Cu(I)-binding properties of the N-terminal soluble domains of *Bacillus subtilis* CopA, Biochem. J. 411 (2008) 571-579.

[24] K.L. Kay, L. Zhou, L. Tenori, J.M. Bradley, C. Singleton, M.A. Kihlken, S. Ciofi-Baffoni, N.E. Le Brun, Kinetic analysis of copper transfer from a chaperone to its target protein mediated by complex formation, Chem. Commun. 53 (2017) 1397-1400.

[25] C. Singleton, N.E. Le Brun, The N-terminal soluble domains of *Bacillus subtilis* CopA exhibit a high affinity and capacity for Cu(I) ions, Dalton Trans. (2009) 688-696.

[26] L. Zhou, C. Singleton, O. Hecht, G.R. Moore, N.E. Le Brun, Cu(I)- and proton-binding properties of the first N-terminal soluble domain of *Bacillus subtilis* CopA, FEBS J. 279 (2012) 285-298.

[27] L. Zhou, C. Singleton, N.E. Le Brun, CopAb, the second N-terminal soluble domain of *Bacillus subtilis* CopA, dominates the Cu(I)-binding properties of CopAab, Dalton Trans. 41 (2012) 5939-5948.

[28] W.L. DeLano, The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, USA, 2002.

[29] L. Banci, I. Bertini, S. Ciofi-Baffoni, M. D'Onofrio, L. Gonnelli, F.C. Marhuenda-Egea, F.J. Ruiz-Duenas, Solution structure of the N-terminal domain of a potential copper-translocating P-type ATPase from *Bacillus subtilis* in the apo and Cu(I) loaded states, J. Mol. Biol. 317 (2002) 415-429.

[30] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMRPipe: a multidimensional spectral processing system based on UNIX pipes, J. Biomol. NMR 6 (1995) 277-293.

[31] N.E. Zhou, B.Y. Zhu, B.D. Sykes, R.S. Hodges, Relationship between amide proton chemical-shifts and hydrogen-bonding in amphipathic alpha-helical peptides, J. Am. Chem. Soc. 114 (1992) 4320-4326.

[32] N. Fatemi, D.M. Korzhnev, A. Velyvis, B. Sarkar, J.D. Forman-Kay, NMR characterization of copper-binding domains 4-6 of ATP7B, Biochemistry 49 (2010) 8468-8477.

[33] C.H. Yu, N.V. Dolgova, O.Y. Dmitriev, Dynamics of the metal binding domains and regulation of the human copper transporters ATP7B and ATP7A, IUBMB Life 69 (2017) 226-235.

[34] Y. Huang, S. Nokhrin, G. Hassanzadeh-Ghassabeh, C.H. Yu, H. Yang, A.N. Barry, M. Tonelli, J.L. Markley, S. Muyldermans, O.Y. Dmitriev, S. Lutsenko, Interactions between metal-binding domains modulate intracellular targeting of Cu(I)-ATPase ATP7B, as revealed by nanobody binding, J. Biol. Chem. 289 (2014) 32682-32693.

[35] C.H. Yu, N. Yang, J. Bothe, M. Tonelli, S. Nokhrin, N.V. Dolgova, L.T. Braiterman, S. Lutsenko, O.Y. Dmitriev, The metal chaperone Atox1 regulates the activity of the human copper transporter ATP7B by modulating domain dynamics, J. Biol. Chem. (2017) DOI: 10.1074/jbc.M117.811752

[36] A.D. Kippen, A.R. Fersht, Analysis of the mechanism of assembly of cleaved barnase from two peptide fragments and its relevance to the folding pathway of uncleaved barnase, Biochemistry 34 (1995) 1464-1468.

[37] F.M. Richards, P.J. Vithayathil, The preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components, J. Biol. Chem. 234 (1959) 1459-1465.

[38] T. Mondol, J. Aden, P. Wittung-Stafshede, Copper binding triggers compaction in N-terminal tail of human copper pump ATP7B, Biochem. Biophys. Res. Commun. 470 (2016) 663-669.

[39] M. Gonzalez-Guerrero, D. Hong, J.M. Arguello, Chaperone-mediated Cu^+ delivery to Cu^+ transport ATPases: requirement of nucleotide binding, J. Biol. Chem. 284 (2009) 20804-20811.

[40] S. Jayakanthan, L.T. Braiterman, N.M. Hasan, V.M. Unger, S. Lutsenko, Human copper transporter ATP7B (Wilson disease protein) forms stable dimers *in vitro* and in cells, J. Biol. Chem. (2017) DOI: 10.1074/jbc.M117.807263.