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Supplementary Information

CopAb, the second N-terminal soluble domain of *Bacillus subtilis* CopA, dominates the Cu(I)-binding properties of CopAab

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Supplementary text

Determination of equilibrium binding constant for Cu(I)-CopAb

The binding of Cu(I) to BCS can be written as:

$$2BCS^{2-} + Cu(I) \leftrightarrow Cu\,(BCS)_2^{3-} \qquad \text{Equ. S1}$$

With the overall formation constant, β_2, given by:

$$\beta_2 = K_1K_2 = \frac{[Cu(BCS)_2^{3-}]}{[BCS^{2-}]^2[Cu(I)]} \qquad \text{Equ. S2}$$

Rearranging to obtain the equilibrium free Cu(I) concentration:

$$[Cu(I)] = \frac{[Cu(BCS)_2^{3-}]}{[BCS^{2-}]^2\beta_2} \qquad \text{Equ. S3}$$

The concentration of free BCS is obtained from total BCS ($[BCS^{2-}]_t$) and $[Cu(BCS)_2^{3-}]$:

$$[BCS^{2-}] = [BCS]_t - (2\times[Cu(BCS)_2^{3-}]) \qquad \text{Equ. S4}$$

Therefore, free Cu(I) is given as:

$$[Cu(I)] = \frac{[Cu(BCS)_2^{3-}]}{\{[BCS]_t - 2\times[Cu(BCS)_2^{3-}]\}^2\beta_2} \qquad \text{Equ. S5}$$

For Cu(I)-binding to CopAb:

$$CopAb + Cu(I) \leftrightarrow CopAb - Cu(I) \qquad \text{Equ. S6}$$

$$K = \frac{[CopAb - Cu(I)]}{[CopAb][Cu(I)]} \qquad \text{Equ. S7}$$

The ratio of bound to free CopAb, [CopAb-Cu(I)]/[CopAb], can be expressed in terms of Cu(I) occupancy, θ, Equ. S8, where $[CopAb]_t$ is the total concentration of bound and unbound CopAb.

$$\theta = \frac{[CopAb - Cu(I)]}{[CopAb]_t} \qquad \text{Equ. S8}$$

Rearranging gives:

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$$[\mathrm{CopAb-Cu(I)}] = \theta[\mathrm{CopAb}]_t \qquad \text{Equ. S9}$$

And,

$$[\mathrm{CopAb}] = (1-\theta)[\mathrm{CopAb}]_t \qquad \text{Equ. S10}$$

Thus,

$$\frac{[\mathrm{CopAb-Cu(I)}]}{[\mathrm{CopAb}]} = \frac{\theta[\mathrm{CopAb}]_t}{(1-\theta)[\mathrm{CopAb}]_t} = \frac{\theta}{1-\theta} \qquad \text{Equ. S11}$$

Substituting this and the expression above for $[Cu(I)]_f$ (Equ. S5) into Equ. S7 gives:

$$K = \frac{\theta}{1-\theta} \cdot \frac{\{[\mathrm{BCS}]_t - 2\times[\mathrm{Cu(BCS)_2}^{3-}]\}^2 \beta_2}{[\mathrm{Cu(BCS)_2}^{3-}]} \qquad \text{Equ. S12}$$

The value of β_2, the formation constant for $[Cu(BCS)_2]^{3-}$, has been determined to be $10^{19.8}$ [1].

pH dependence of the equilibrium binding constant for Cu(I)-CopAb

In order to determine the true equilibrium binding constant, the effects of competition between protons and Cu(I) need to the considered. These could be direct competition effects, where, protons and Cu(I) compete for cysteine thiolates, or indirect, where protonation of a non-cysteine residue inhibits Cu(I)-binding at the Cu(I) binding motif. For a single protonation/deprotonation event, we can write:

$$\mathrm{CopAb}^n + \mathrm{H}^+ \leftrightarrow \mathrm{CopAbH}^{n+1} \qquad \text{Equ. S13}$$

$$K_{H^+} = \frac{[\mathrm{CopAbH}^{n+1}]}{[\mathrm{CopAb}^n][\mathrm{H}^+]} \qquad \text{Equ. S13}$$

The concentration of deprotonated CopAb present is given below (Equ. S14), where $[CopAb]_{f,t}$ is the total concentration of unbound CopAb:

$$[\mathrm{CopAb}^n] = \alpha[\mathrm{CopAb}]_{f,t} \qquad \text{Equ. S14}$$

Here, α is the fraction of CopAb that is deprotonated:

$$\alpha = \frac{[\mathrm{CopAb}^n]}{[\mathrm{CopAb}^n] + [\mathrm{CopAbH}^{n+1}]} \qquad \text{Equ. S15}$$

From the equilibrium expression, Equ. S13:

$$\alpha = \frac{[\mathrm{CopAb}^n]}{[\mathrm{CopAb}^n] + [\mathrm{CopAb}^n]K_{\mathrm{H}^+}[\mathrm{H}^+]} \qquad \text{Equ. S16}$$

This reduces to:

$$\alpha = \frac{1}{1 + K_{H^+}[\mathrm{H}^+]} \qquad \text{Equ. S17}$$

And so:

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$$[\mathrm{CopAb^n}] = \frac{[\mathrm{CopAb}]_{t}}{1 + K_{H^+}[\mathrm{H^+}]} \qquad \text{Equ. S18}$$

Substituting this into Equ. S7 gives:

$$K = \frac{[\mathrm{CopAb\text{-}Cu(I)}]}{(\frac{[\mathrm{CopAb}]_{t}}{1 + K_{H^+}[\mathrm{H^+}]})[\mathrm{Cu(I)}]} \qquad \text{Equ. S19}$$

Which is the same as:

$$K = \frac{[\mathrm{CopAb\text{-}Cu(I)}](1 + K_{H^+}[\mathrm{H^+}])}{[\mathrm{CopAb}]_{t}[\mathrm{Cu(I)}]} \qquad \text{Equ. S20}$$

Here, $(1+K_{H^+}[H^+])$ can be considered as a correction factor for the value of K determine without taking into account the effect of proton competition [2]. The true value of K, for a single proton competition process, can be represented as a pH-dependent-component multiplied by a correction factor.

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Supplementary Tables

Table S1. pH-dependence of Cu(I)-binding to CopAb.

	pH 7.0				pH 7.5				pH 8.0			
$[CopAb]_t$ μM	30[a]	27.3[b]	30[a]	27.3[b]	30[a]	27.3[b]	30[a]	27.3[b]	30[a]	27.3[b]	30[a]	27.3[b]
$[BCS]_t$ mM	0.75	0.68	0.50	0.45	0.75	0.68	0.50	0.45	0.75	0.68	0.50	0.45
$[Cu(I)]_t$ μM	10	10	10	10	10	10	10	10	10	10	10	10
A_{483nm}	0.121	0.103	0.111	0.097	0.089	0.079	0.066	0.054	0.079	0.050	0.052	0.047
$[Cu(bcs)_2^{3-}]$ μM	9.09	7.47	8.37	7.31	6.72	5.97	4.97	4.04	5.96	3.76	3.89	3.53
Cu occupancy, θ[c]	0.03	0.082	0.054	0.098	0.11	0.15	0.17	0.22	0.14	0.23	0.20	0.24
$[Cu(I)]_f$, 10^{-19}M	2.69	2.78	5.68	6.11	1.93	2.12	3.28	3.22	1.73	1.32	2.54	2.85
K **(× 10^{17}) M^{-1} [d]**	1.15	3.22	1.01	1.78	6.41	8.13	6.10	8.77	9.43	22.7	9.90	11.1
Average *K* (× 10^{17}) M^{-1} [e]	1.79 ± 1.01				7.35 ± 1.30				13.28 ±6.32			

[a] Data resulting from addition of BCS to CopAb. The buffer used was 100 mM Mops and 100 mM NaCl, at the indicated pH.
[b] Data resulting from addition of CopAb to BCS.
[c] From Equ. S8.
[d] From Equ. 2.
[e] Indicated error is ±SD.

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Supplementary Figures

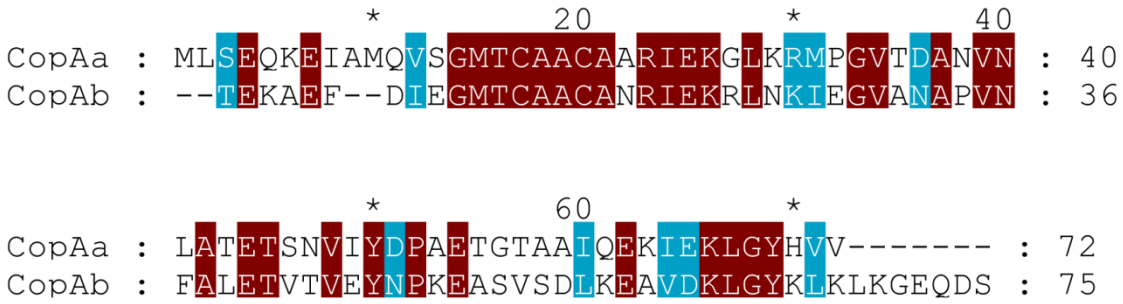

Figure S1 Amino acid residue sequence alignment of *B. subtilis* CopAa with CopAb. Numbers at the right of the figure indicate the amino acid residue number relative to the start of each polypeptide sequence. Residues highlighted in red are conserved, while those in turquoise are conservatively substituted.

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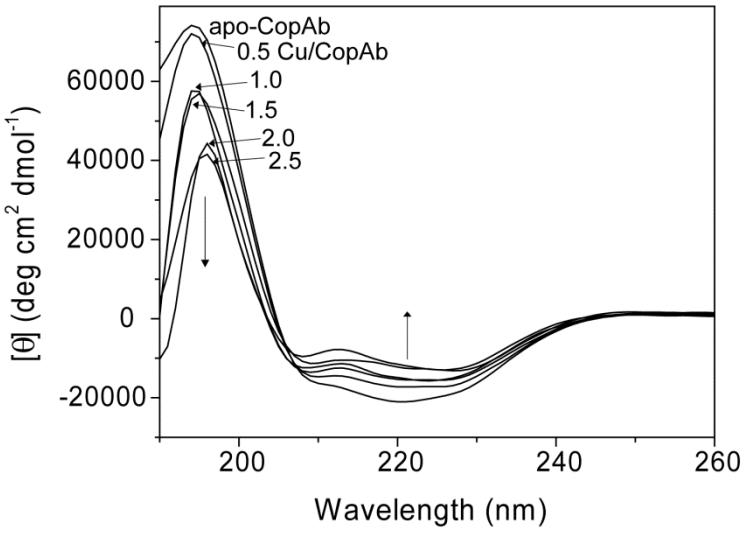

Figure S2. Cu(I)-mediated CopAb structural changes monitored by CD spectroscopy. Far-UV CD spectra of CopAb following the addition of 0 – 2.5 Cu(I) ions per protein molecule, as indicated. CopAb (15 μM) was in 50 mM phosphate, pH 7. The spectra show that initial binding of Cu(I) does not affect secondary structure content. At increasing Cu(I) loadings, some intensity is lost, consistent with loss of some β-sheet content. However, even at 2.5 Cu(I) per protein, a large component of the original secondary structure remains.

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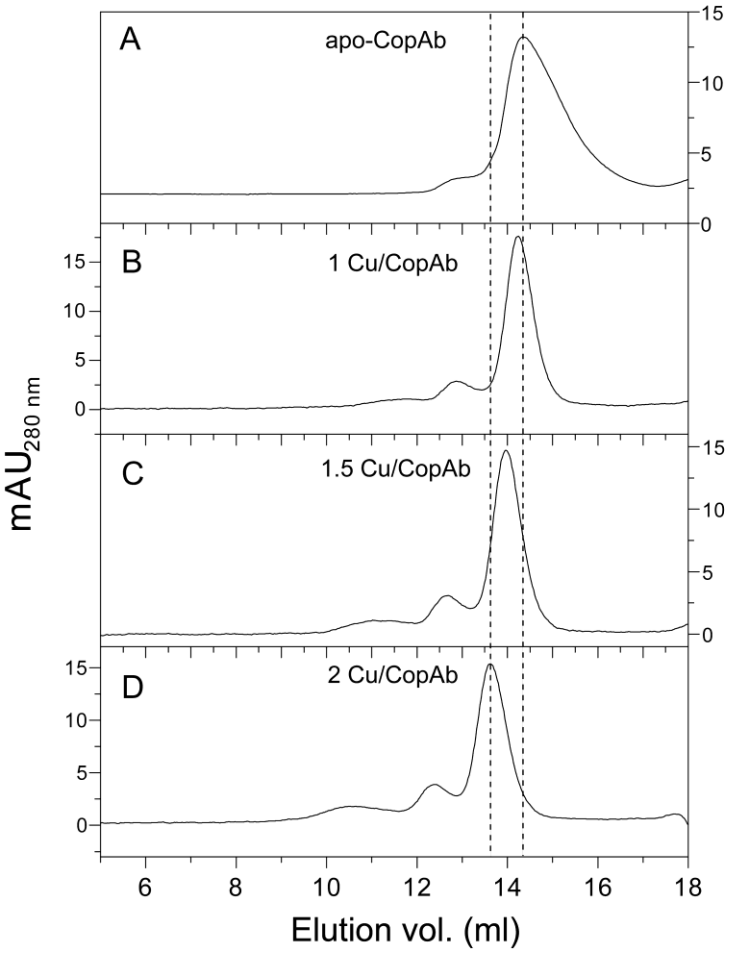

Fig. S3. Cu(I)-mediated CopAb association state changes monitored by gel flitration. Analytical gel filtration chromatographs of apo-CopAb and Cu(I) bound forms, as indicated. Plots show intensity at 280 nm as a function of elution volume. CopAb (95 μM) was in 100 mM Mops, 100 mM NaCl, pH 7.5. In (A), apo-CopAb gave a peak at elution volume 14.4 ml, with a minor shoulder at 13 ml. In (B), addition of 1 Cu(I) per protein also resulted in a major peak at 14.4 ml, with a shoulder at 13 ml, though the major peak was significantly less broad. This indicates that the protein did not alter its association state upon binding a single Cu(I) ion. In (C), when the loading of Cu(I) was increased up to 1.5 Cu(I) per CopAb, the peak shifted to 14.0 ml, while, in (D), at 2.0 Cu(I) per CopAb, the elution peak was observed at 13.5 ml. In both (C) and (D), a minor band was again observed, and these were also shifted compared to the equivalent band in (B). The data indicate that CopAb aggregation occurs above 1.0 Cu(I)/CopAb. The minor bands running ahead of the major eluting species arise from a small proportion of CopAb that has aggregated to higher molecular mass forms. That these species also shift upon addition of Cu(I) suggests that they are also able to bind Cu(I). These data are in generally good agreement with the AUC data presented in the main paper. The only difference of note relates to a loading of 1.5 Cu(I) per protein. While the AUC data indicate that the protein is a dimer, the gel filtration data suggest that, with an elution volume between the monomer and dimer, the protein is likely to be in rapid equilibrium between these two forms. The reason for this difference is not clear, but may reflect that gel filtration is not a true equilibrium method (because it is a separation technique). Nevertheless, both methods indicate that protein association occurs above a level of 1 Cu(I) per CopAb protein.

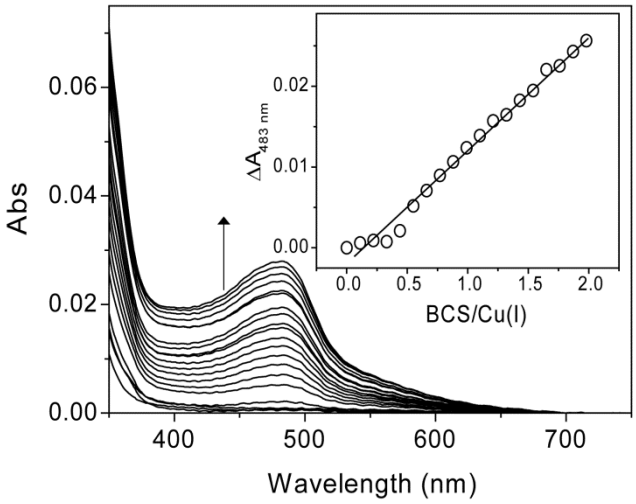

Figure S4. Competition between CopAb and the chelator BCS for Cu(I). UV-visible spectra recorded following additions of BCS into CopAb (24 μM) containing 12 μM Cu(I) in 100 mM Mops, 100 mM NaCl, pH 7.5. Inset is a plot of changes in $A_{483\ nm}$ during the course of the titration.

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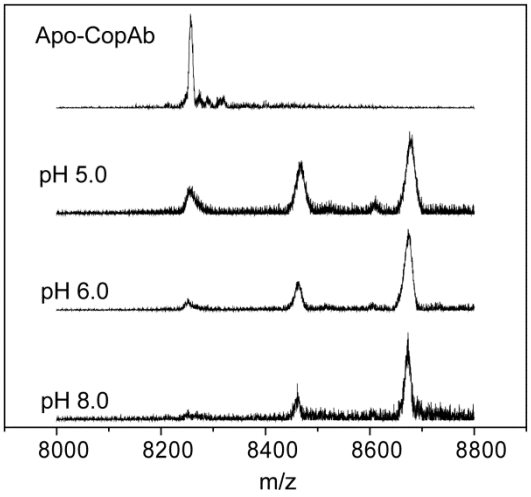

Figure S5. Investigation of CopAb Cys residue reactivity as a function of pH using MALDI-TOF. MALDI-TOF spectra of samples of apo-CopAb (at pH 7) before (control) and following the addition of apo-CopAb (1 μM) to badan (13 μM) at different pH values, as indicated. Mass spectra were recorded using a Shimadzu Biotech AXIMA- CFR instrument; samples were prepared with alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix.

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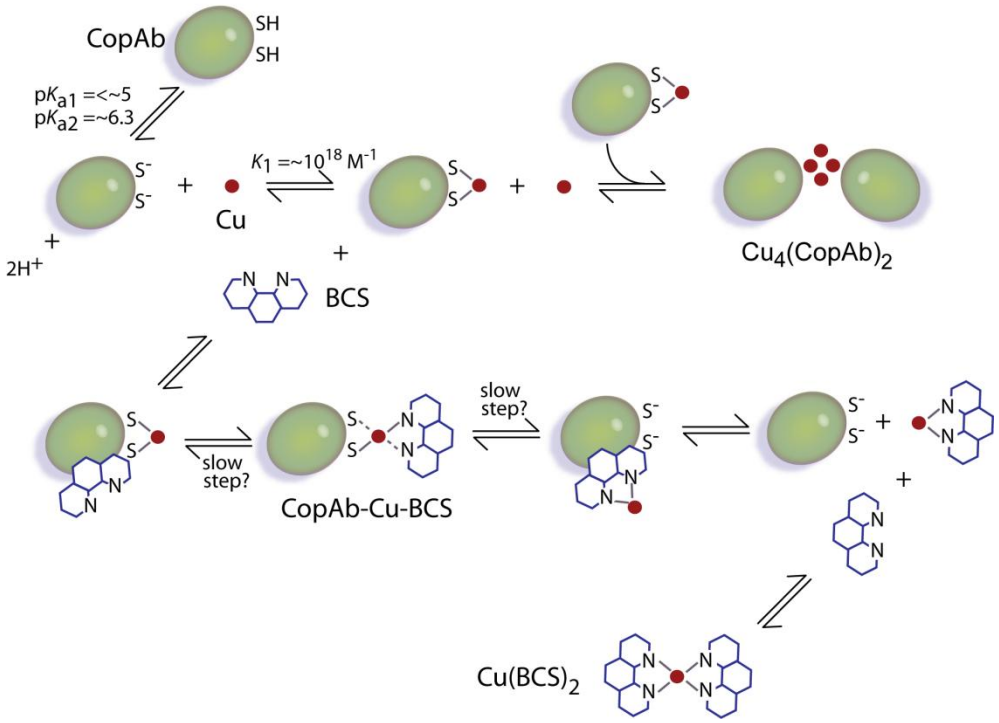

Figure S6. Schematic diagram of proton and Cu(I) binding to CopAb and the mechanism of Cu(I) exchange between CopAb and BCS. CopAb contains two cysteine residues with active sites giving pK_{a1} of < 5 and pK_{a2} of ~ 6.3. CopAb is able to initially bind Cu(I) as monomer complex with high affinity (pH corrected K_{eq} of ~ 10^{18} M^{-1}). Once an excess of BCS is added to Cu(I) loaded CopAb, Cu(I) exchange occurs rapidly. The rate-determining step likely corresponds to the breakdown of a CopAb-Cu(I)-BCS intermediate; this is supported by the measurements of identical rate same rate constants for the two directions of the exchange. In the case of addition of apo-CopAb to $Cu(BCS)_2$, an initial rapid reaction occurs before the rate determining slow step, which could, for example, correspond to the formation of the hetero protein-Cu(I)-chelator complex.

References

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2. L. Zhou, C. Singleton and N. E. Le Brun, *Biochem. J.*, 2008, **413**, 459-465.