Electronic Supplementary Material (ESI) for Metallomics.


# The pathways and domain specificity of Cu(I) binding to human metallothionein 1A: Electronic Supplementary Information

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**Amino acid sequences of βα MT and the β and α domain fragments**

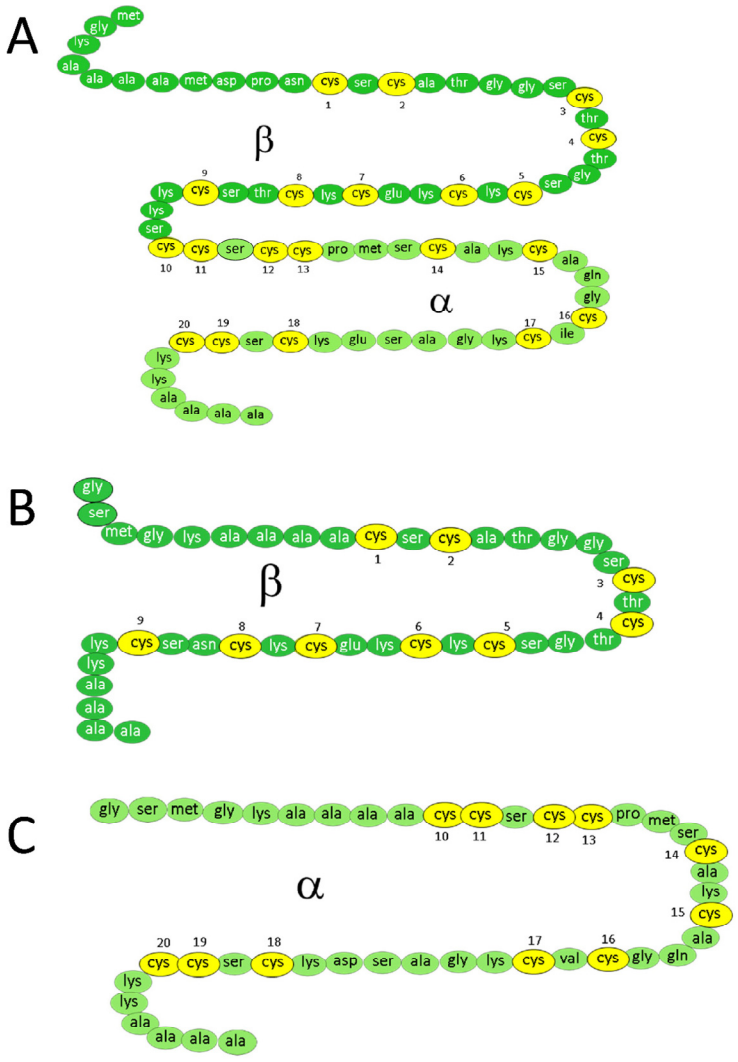


*Fig. S1* ***A:*** *Amino acid sequence of recombinant human MT1A with cysteine residues shown in yellow. When metallated with a total of 7 zinc or cadmium, protein folding results in metal binding domains in the β and α regions.* ***B:*** *Amino acid sequences for the isolated 9 cysteine β fragment.* ***C****: Amino acid sequence for the isolated 11 cysteine α fragment. Note that as a result of the expression system, four alanine residues are added to the N and C termini.*

**Non-cooperative and cooperative binding**

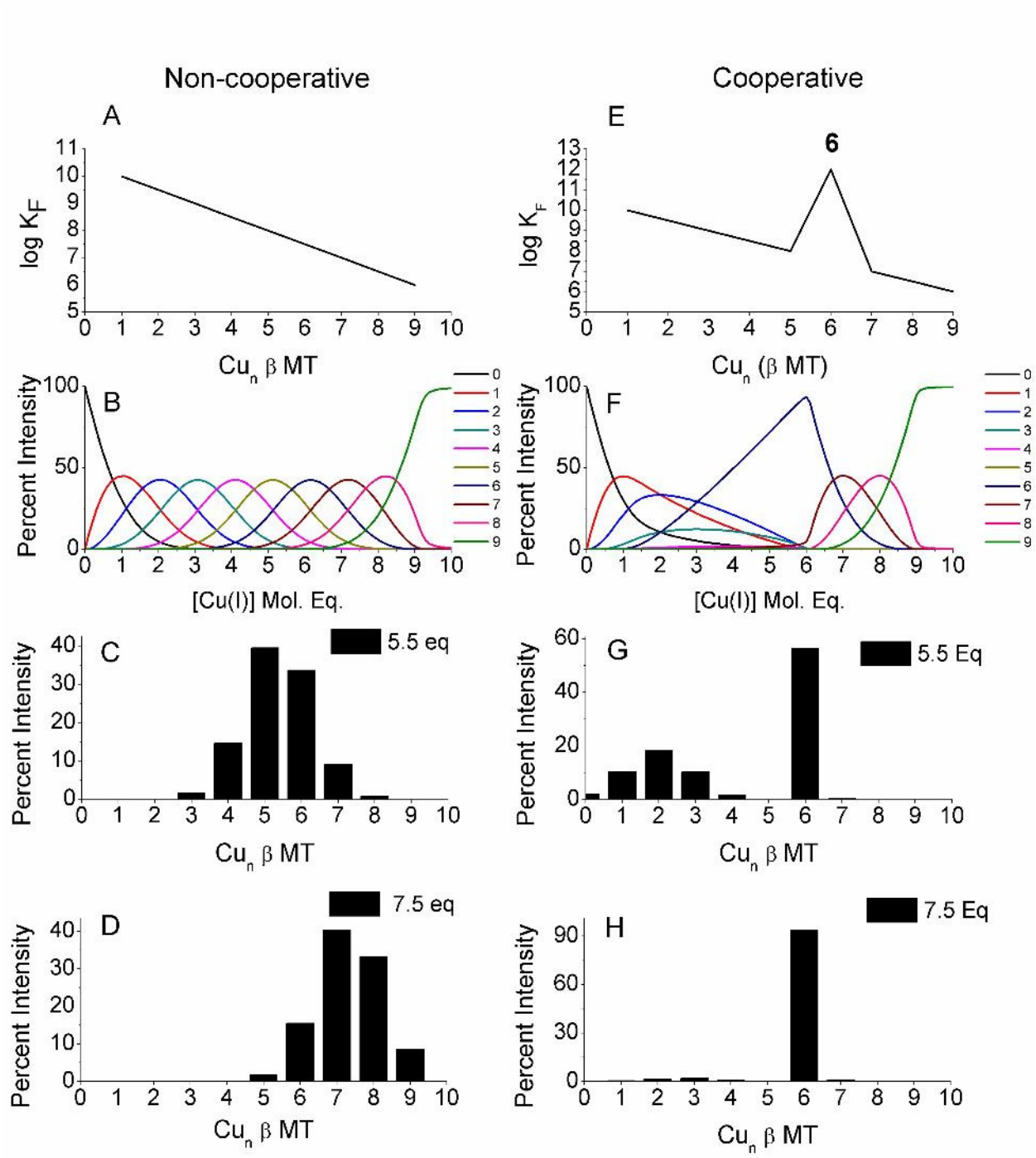


*Fig. S2 Simulation model of non-cooperative and cooperative binding of Cu(I) to the β domain of MT1A. A: Simulated log $K_F$ values decreasing in non-cooperative fashion for each subsequent Cu(I) addition (10, 9.5, 9, 8.5, 8, 7.5, 7, 6.5, 6). B: Simulated speciation graph corresponding to non-cooperative log $K_F$ values shown in A. C: Simulated mass spectrum for non-cooperative binding at 5.5 mol. eq. of Cu(I) added. D: Simulated mass spectrum for non-cooperative binding at 7.5 mol. eq. of Cu(I) added. E: Simulated log $K_F$ values for cooperative binding of $Cu_6S_9$ (10, 9.5, 9, 8.5, 8, 12, 7, 6.5, 6). F: Simulated speciation graph showing effect of cooperative log $K_F$ for $Cu_6S_9$ formation shown in F. G: Simulated mass spectrum for cooperative binding at 5.5 mol. Eq. of Cu(I) added. H: Simulated mass spectrum for cooperative binding at 7.5 mol. eq. of Cu(I) added.*

**Mass Spectral Deconvoluted Spectra and charge State Data: βα MT**

Select deconvoluted ESI-mass spectra of the Cu(I) titration of βα MT are shown in Fig. S3 with the charge state data used to generate the deconvoluted spectra shown in Fig. S4. All measurements were carried out using a Bruker MicrOTOF II (Bruker Daltonics) in positive ion mode. The following parameters were used for all ESI-mass spectral measurements: capillary voltage= 4200V, Nebulizer=2.0 Bar, Dry gas=4.0 L/min, Dry Temp= 200 °C. Samples were introduced by direct infusion

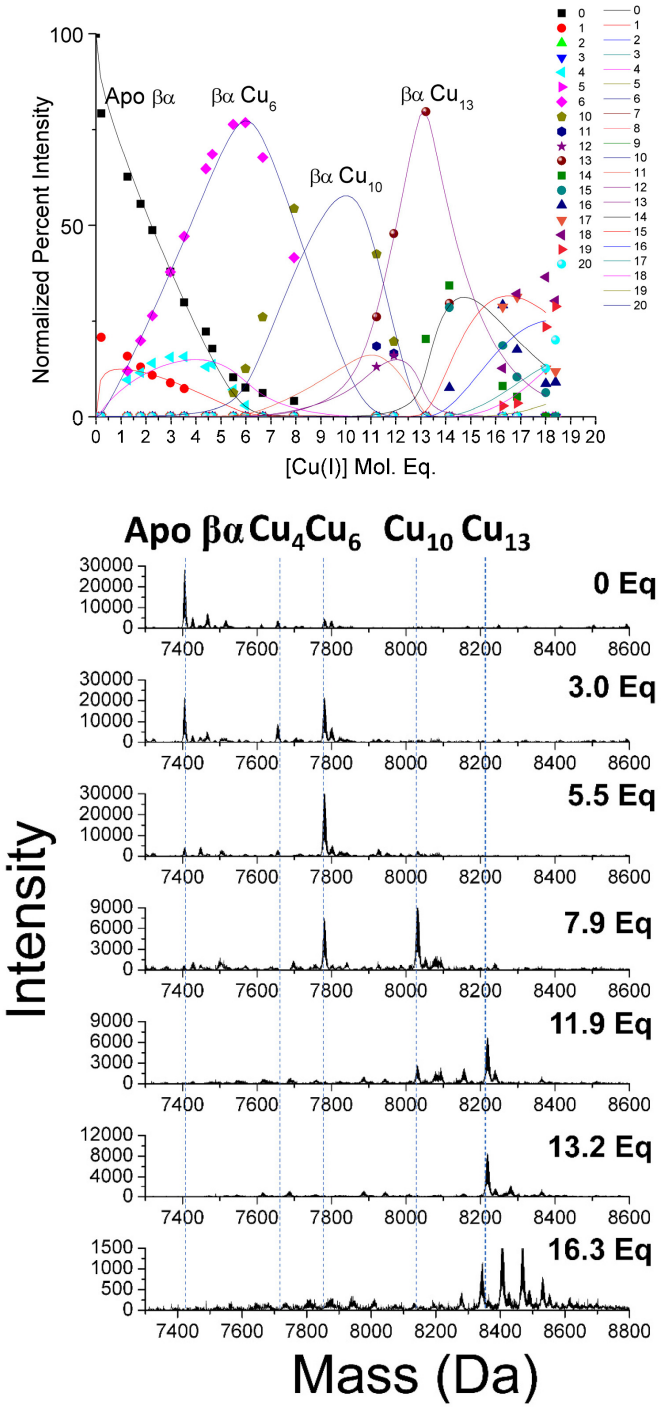


*Fig. S3 Stepwise metallation of apo βα MT at pH 7.4. The protein concentration was 56.2 µM. Molar equivalents of the Cu(I) solution were titrated into the apo protein at room temperature. The Cu(I) stoichiometry in the clustered species that form sum to the totals shown above each major species, for example, $Cu_6$ is associated with $Cu_6S_9$, $Cu_{10}$ arises from two clusters: $Cu_6S_9$ and $Cu_4S_6$, and $Cu_{13}$ arises from two clusters: $Cu_6S_9$ and $Cu_7S_9$.*

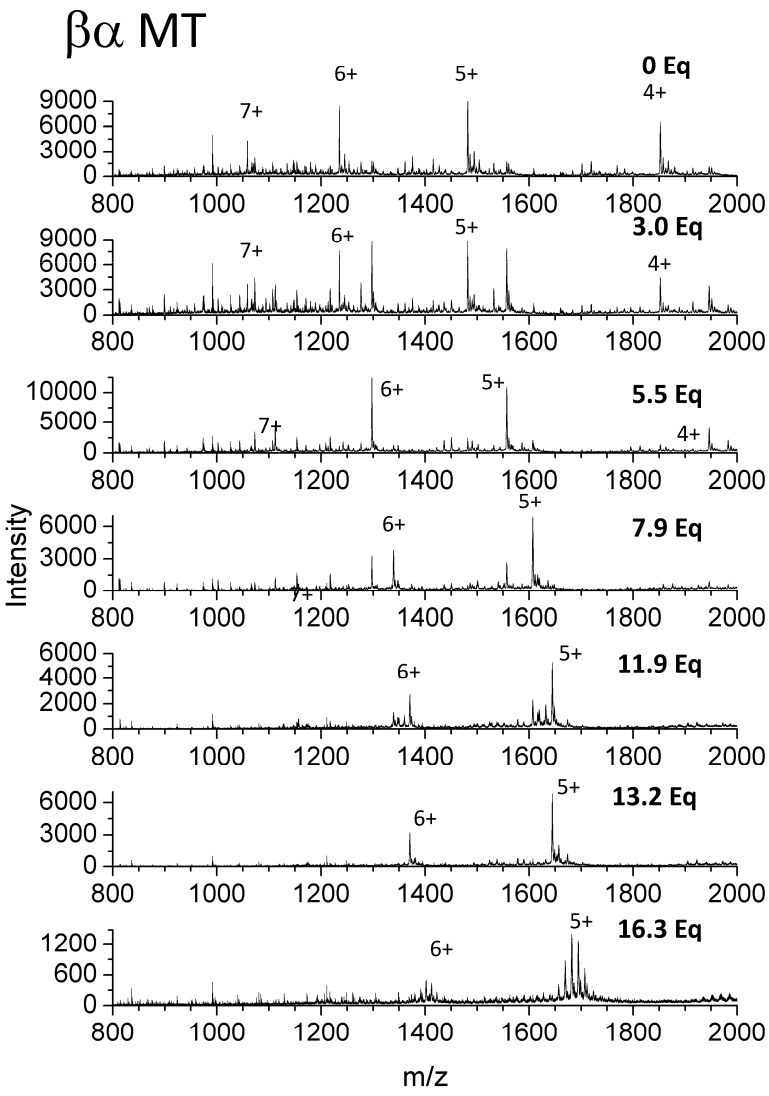


*Fig. S4 Charge state data for Cu(I) titration into apo βα MT at pH 7.4 and room temperature.*

**Mass Spectral Deconvoluted spectra and charge State Data: β domain fragment**

Deconvoluted spectra at select points in the Cu(I) titration of the apo β domain fragment are shown in Fig. S5 with the corresponding charge state data in Fig. S6.

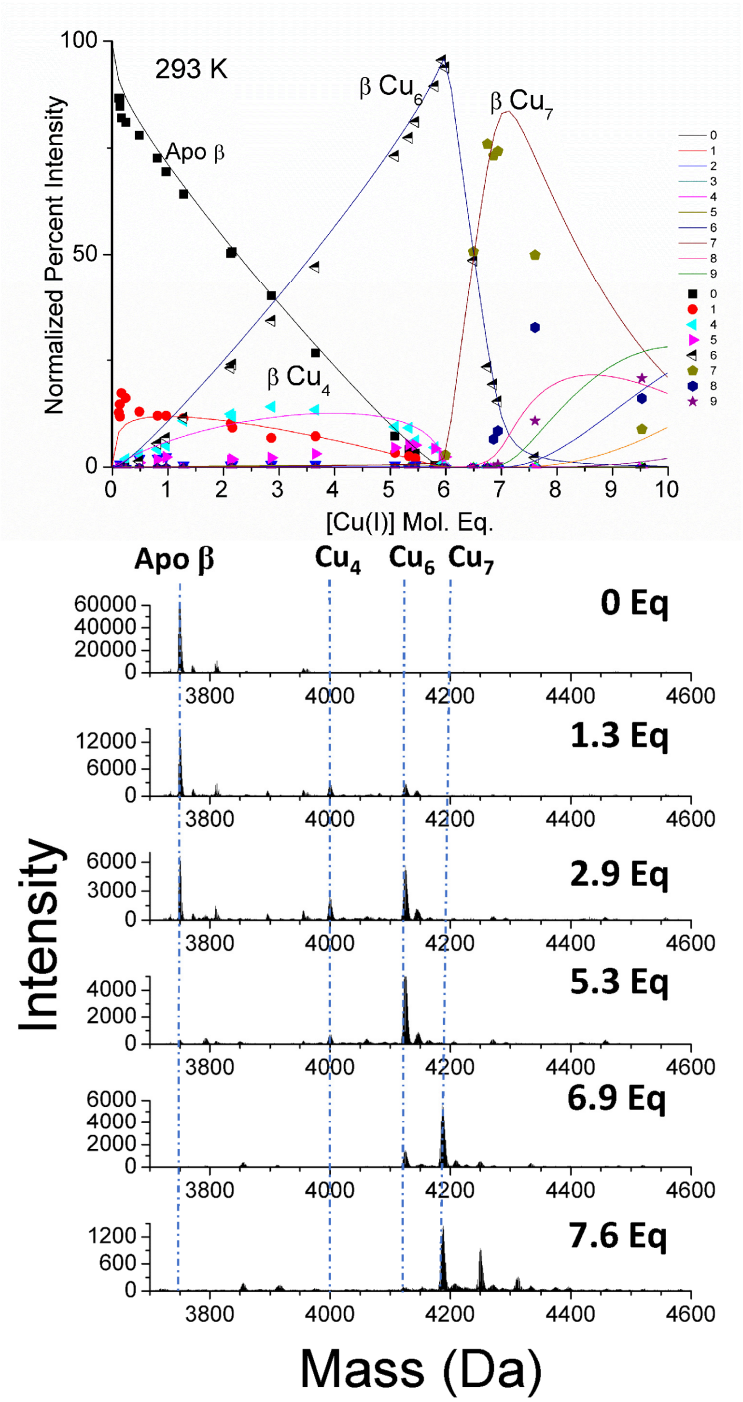


*Fig. S5 Stepwise Cu(I) titration into the apo β domain fragment at pH 7.4. Protein concentration was 156 μM. Molar equivalents of Cu(I) were added at room temperature.*

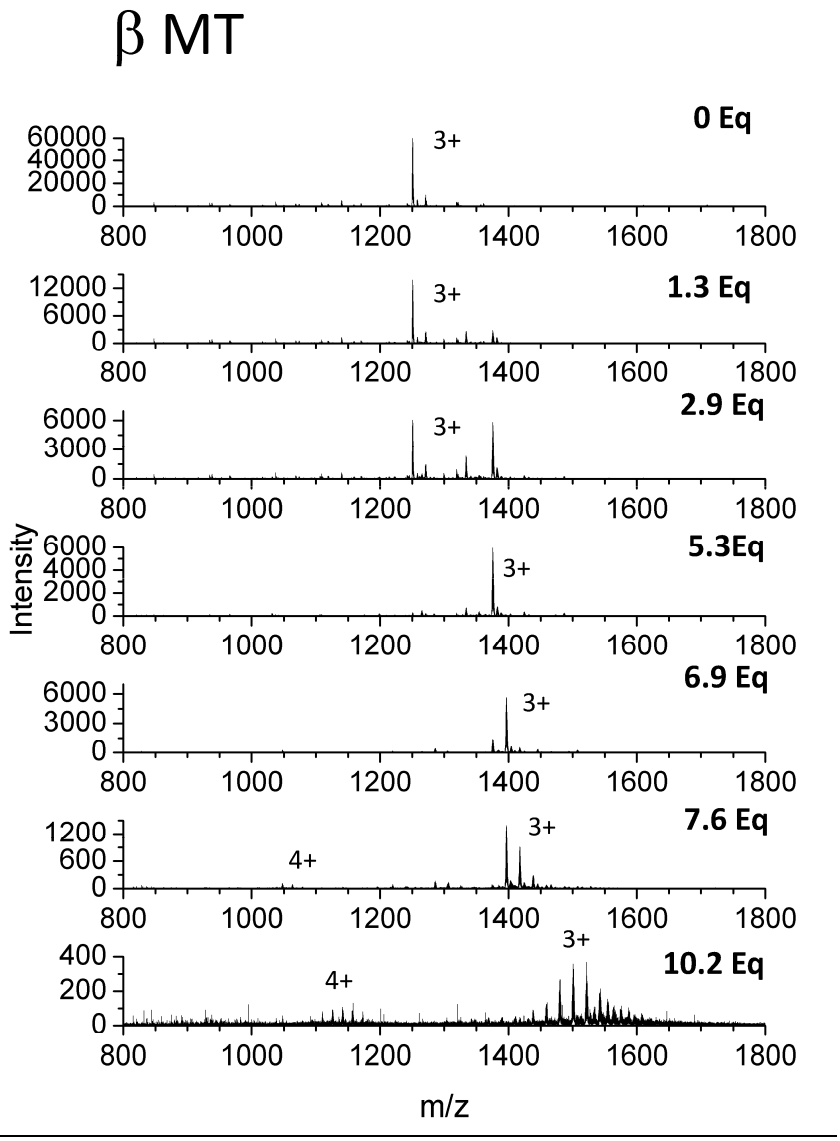


*Fig. S6 Mass spectral charge state data for the titration of Cu(I) into the apo β domain fragment at pH 7.4 and room temperature.*

**Metallation of β MT at 50°C**

The temperature dependence of the metallation of the β domain fragment was tested by metallating the protein at 50 °C (Fig. S7). A solution of the apo β domain fragment and the 10mM Cu(I) solution were kept in a water bath set to 50 °C. The hot Cu(I) solution was added to the protein and allowed to equilibrate for 10 minutes before measuring via ESI-MS. A special thermostatically regulated pump was used to keep heat loss upon injecting the solution into the MS to a minimum.

The resulting metal speciation was very similar to the room temperature data. The increased temperature resulted in slightly more $Cu_4$ forming, however $Cu_6$ and $Cu_7$ were still the major products.

Interestingly, the sharp transition from $Cu_6$ to $Cu_7$ at ~ 7 mol. eq. was not affected by the higher temperature.

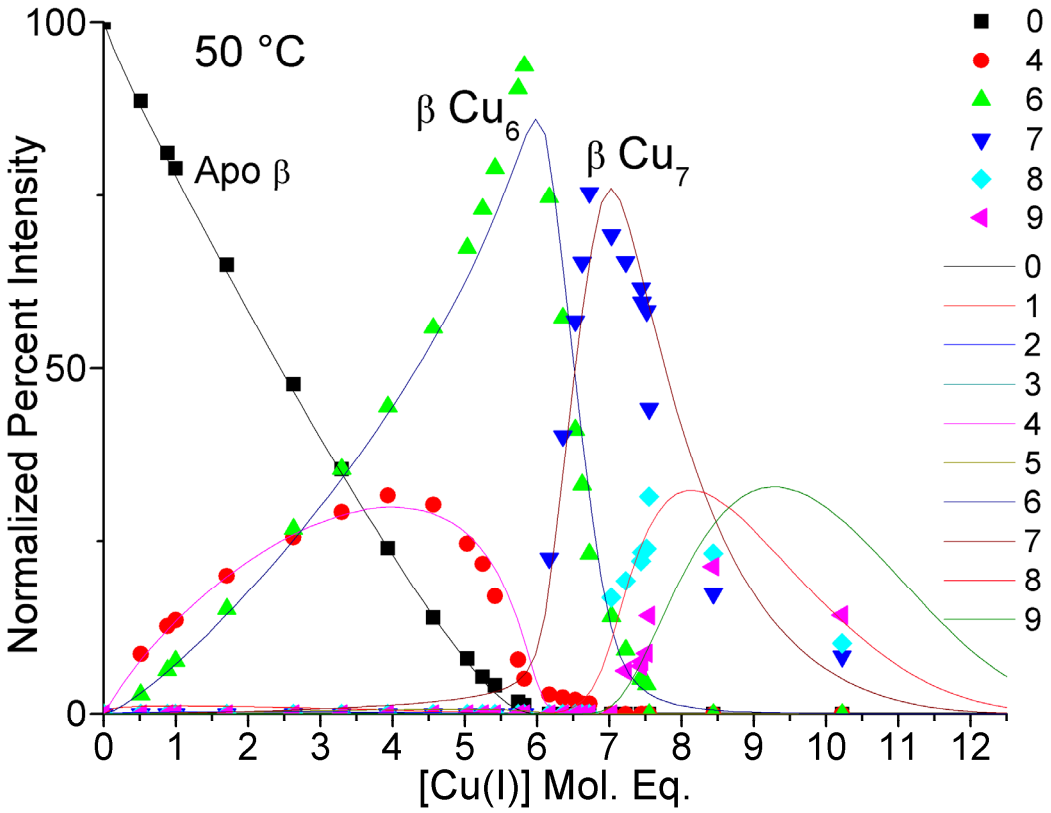


*Fig. S7 Experimental (symbols) and simulated speciation (continuous lines) of the Cu(I) titration into the apo β domain fragment at pH 7.4 and 50 °C*

**Mass Spectral charge State Data: α domain fragment**

Deconvoluted spectra at select points in the Cu(I) titration of the apo α domain fragment are shown in Fig. S8 with the corresponding charge state data in Fig. S9.

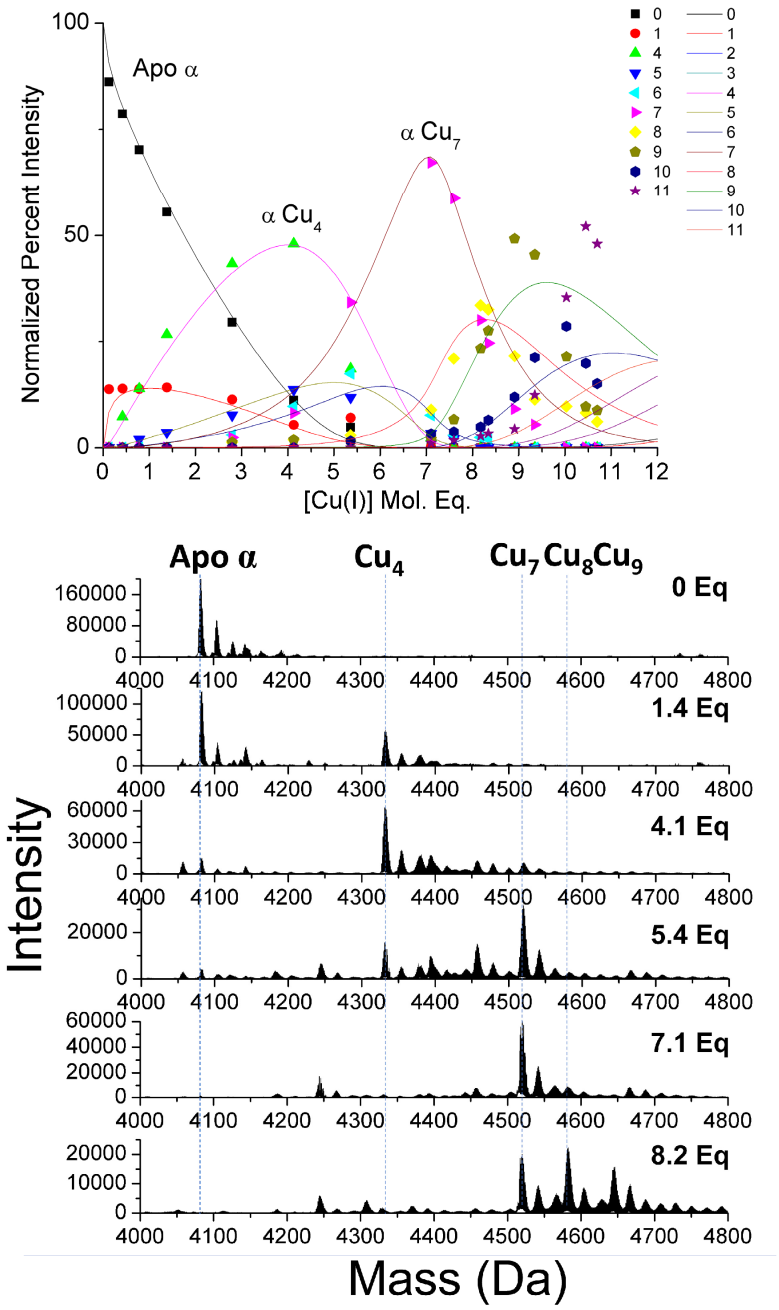


*Fig. S8 Deconvoluted ESI-mass spectra showing the room temperature stepwise copper titration into a 55 μM solution of the apo α domain fragment at pH 7.4.*

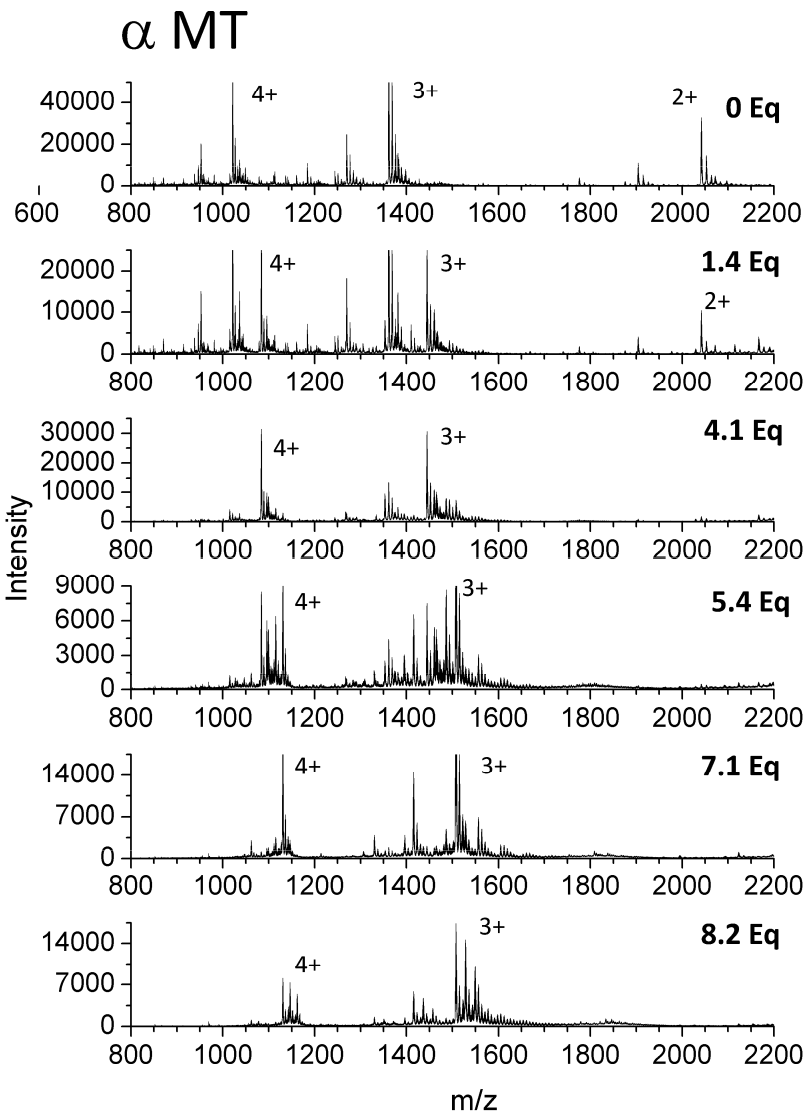


*Fig. S9 Mass spectral charge state data for the Cu(I) titration into the apo α domain fragment at pH 7.4 and room temperature.*

**Benzoquinone studies reveal Cu(I)-thiolate cluster stoichiometries**

Benzoquinone (BQ) was added to solutions of metallated β or α domain fragments to determine the number of free cysteine residues. The Cu(I)-MT-BQ products were measured using ESI-MS. BQ was added to a solution of the β domain fragment metallated with 0, 4, or 6 Cu(I) ions (Fig. S10A). A total of 9 BQ molecules bound to the apo β domain fragment as expected, demonstrating that none of the 9 thiols were binding metals prior to the BQ addition. The $Cu_4$ cluster in the β domain fragment bound 3 BQ molecules, indicating that the cluster stoichiometry is $Cu_4S_6$. Finally, the $Cu_6$ cluster did not allow for any BQ molecules to bind indicating that all 9 thiols are used to form the $Cu_6S_9$ cluster.

Addition of BQ to a mixture of $Cu_4$, $Cu_5$, $Cu_6$, and $Cu_7$ formed in the α domain fragment indicated the number of thiols used to form each Cu(I) species in the α domain fragment (Fig. S10B). The $Cu_4$ cluster in the α domain fragment bound a maximum of 5 BQ molecules, indicating that the cluster stoichiometry is $Cu_4S_6$. The $Cu_7$ cluster bound 2 BQ molecules indicating that that cluster stoichiometry is $Cu_7S_9$. The intermediate products, $Cu_5$ and $Cu_6$ between the two major clusters bind using 7 and 8-9 thiols respectively. One thiol in the $Cu_6$ species may be bound loosely, allowing for a small fraction of the $Cu_6$ species to bind 3 BQ molecules.

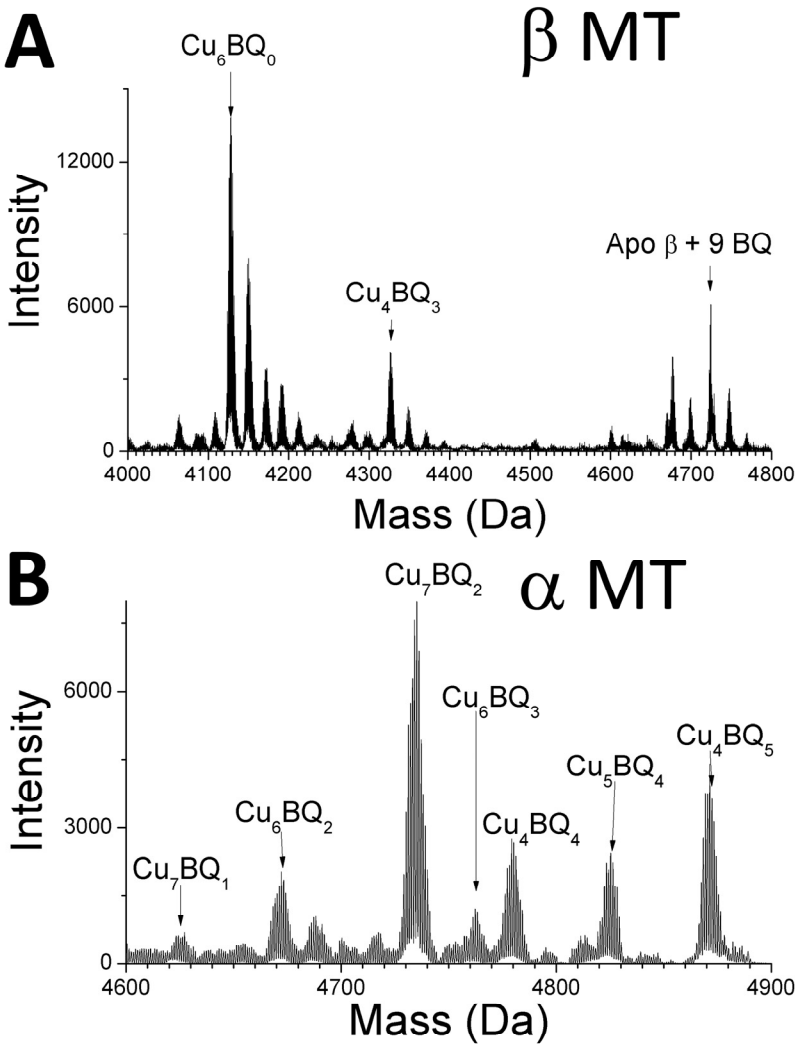


*Fig. S10 ESI-mass spectral data recorded for separate solutions of partially metallated 140 µM α domain fragment (A) and 61 µM β (B) domain fragment in which benzoquinone was added until no further change in the spectra were observed, showing the number of free cysteines for each Cu(I):MT species. A: ESI-mass spectral data showing the addition of BQ to a mixture of apo, $Cu_4$, and $Cu_6$ clusters in the β domain fragment formed at room temperature. B: ESI-mass spectral data showing the addition of BQ to a mixture of $Cu_4$, $Cu_5$, $Cu_6$, and $Cu_7$ in the α domain fragment at room temperature.*

**Copper clusters forming in the two domains of MT have different phosphorescent lifetimes**

The apo β domain fragment was metallated with Cu(I) at pH 7.4 and room temperature. The speciation was confirmed using ESI-MS. The decay of the emission after the flash lamp pulse of ~ 10µs is shown in Fig. S11 for the clusters forming in the β domain fragment. The $Cu_6$ cluster emission had a lifetime of 2.66 ±.02 µs. The sample was excited at 280nm and the emission at 710 nm was monitored. For the $Cu_7$ cluster, the sample was again excited at 280 nm, but its emission was monitored at 633 nm. This cluster had a longer lifetime of 5.74 ± .03µs.

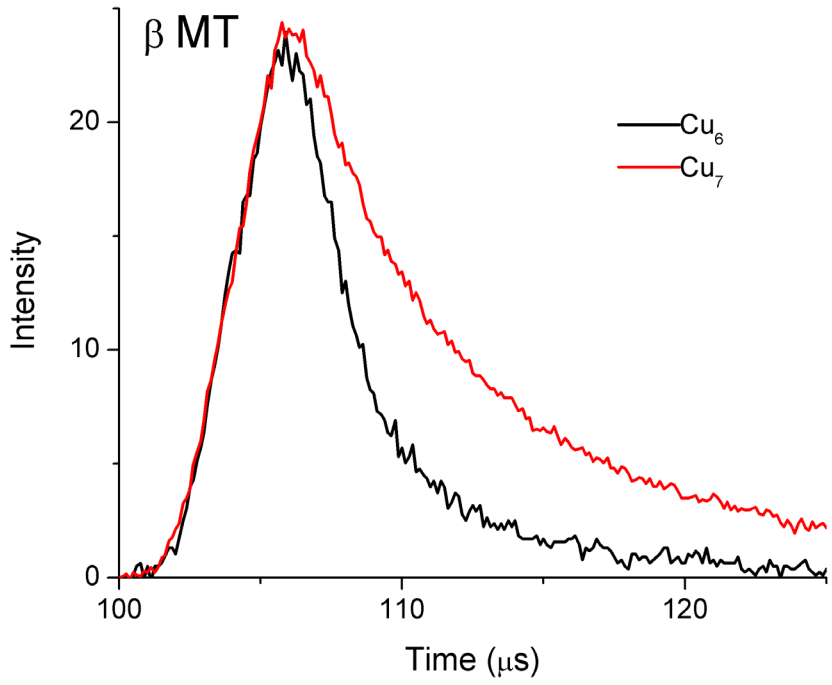


*Fig. S11 Lifetime data for the significant clusters formed in the β domain fragment: $Cu_6$ and $Cu_7$*

The α domain fragment was metallated at pH 7.4 and room temperature with Cu(I) to form $Cu_4$ and $Cu_7$. At each copper loading, the solution was measured by ESI-MS to determine the speciation. The lifetimes for the $Cu_4$ and $Cu_7$ clusters formed in the α domain fragment were monitored by exciting at 280 nm for both and measuring the emission at 630 nm and 634 n, respectively (Fig. S12)

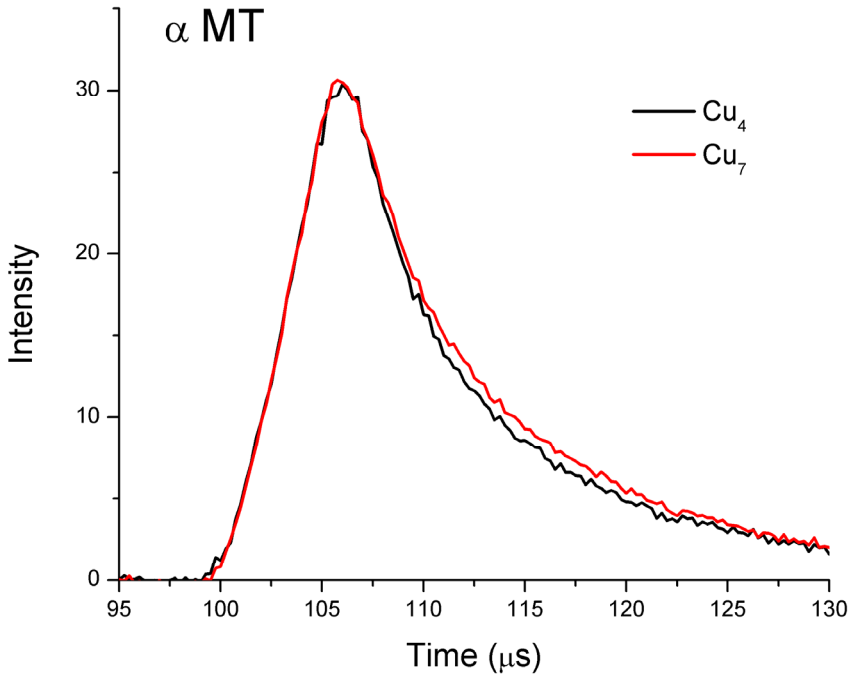


*Fig. S12 Lifetime data for the $Cu_4$ and $Cu_7$ clusters formed in the α domain fragment.*

**Competitive Binding between β and α domain fragments**

Fig. S13 shows select deconvoluted ESI-mass spectra for various points in the Cu(I) titration into equimolar amounts of the apo β and apo α domain fragments. The many species in solution make the deconvoluted spectra more informative than the charge state data.

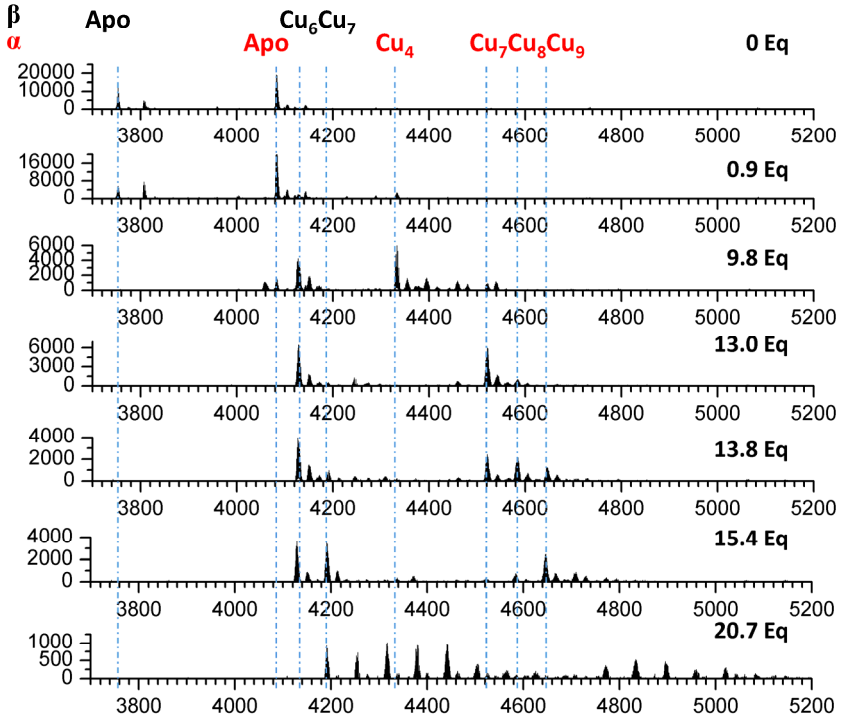


*Fig. S13 Deconvoluted ESI-mass spectral data for the titration of Cu(I) into an equimolar mixture of the β and α domain fragments at pH 7.4 and room temperature.*

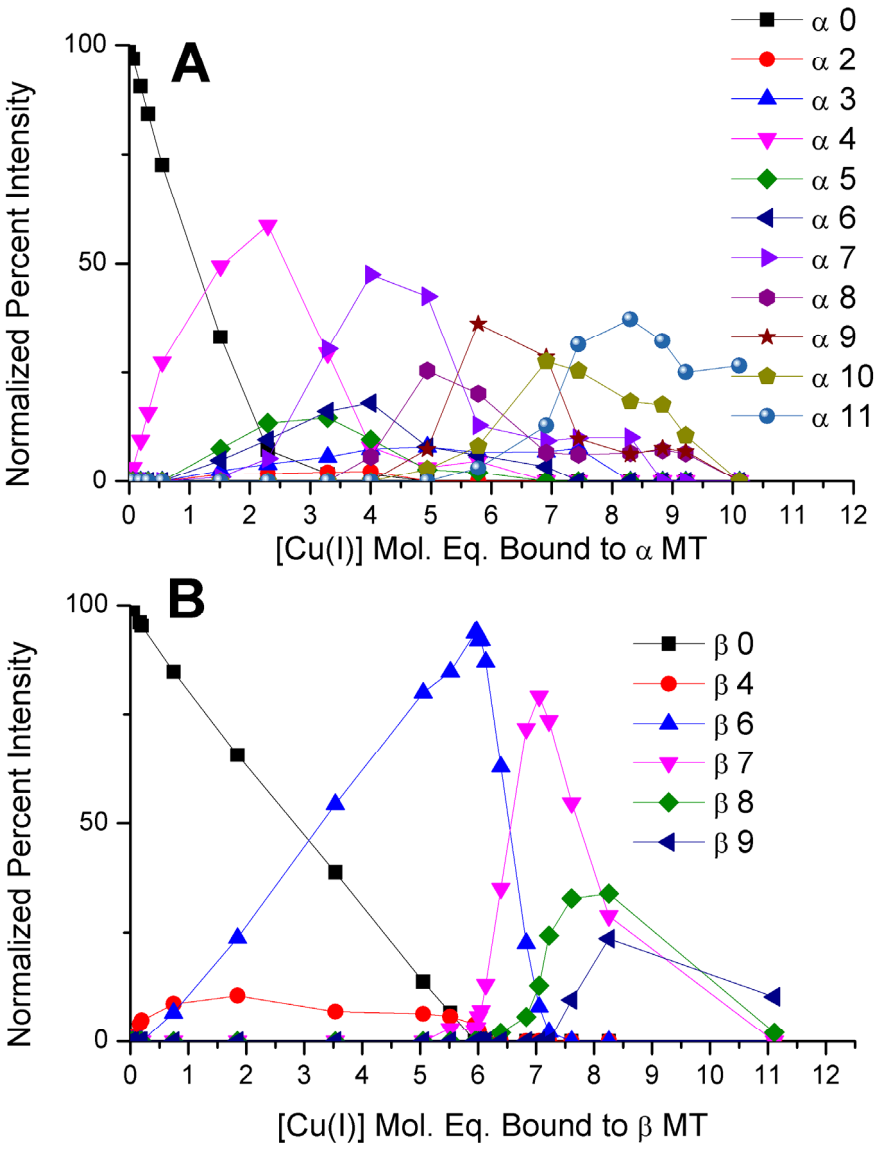


*Fig. S14 Cu(I) speciation following titration of Cu(I) into solution of both apo α and apo β domain fragments with the x- axis showing the amount of Cu(I) bound to A: α domain fragment and B: the β domain fragment.*

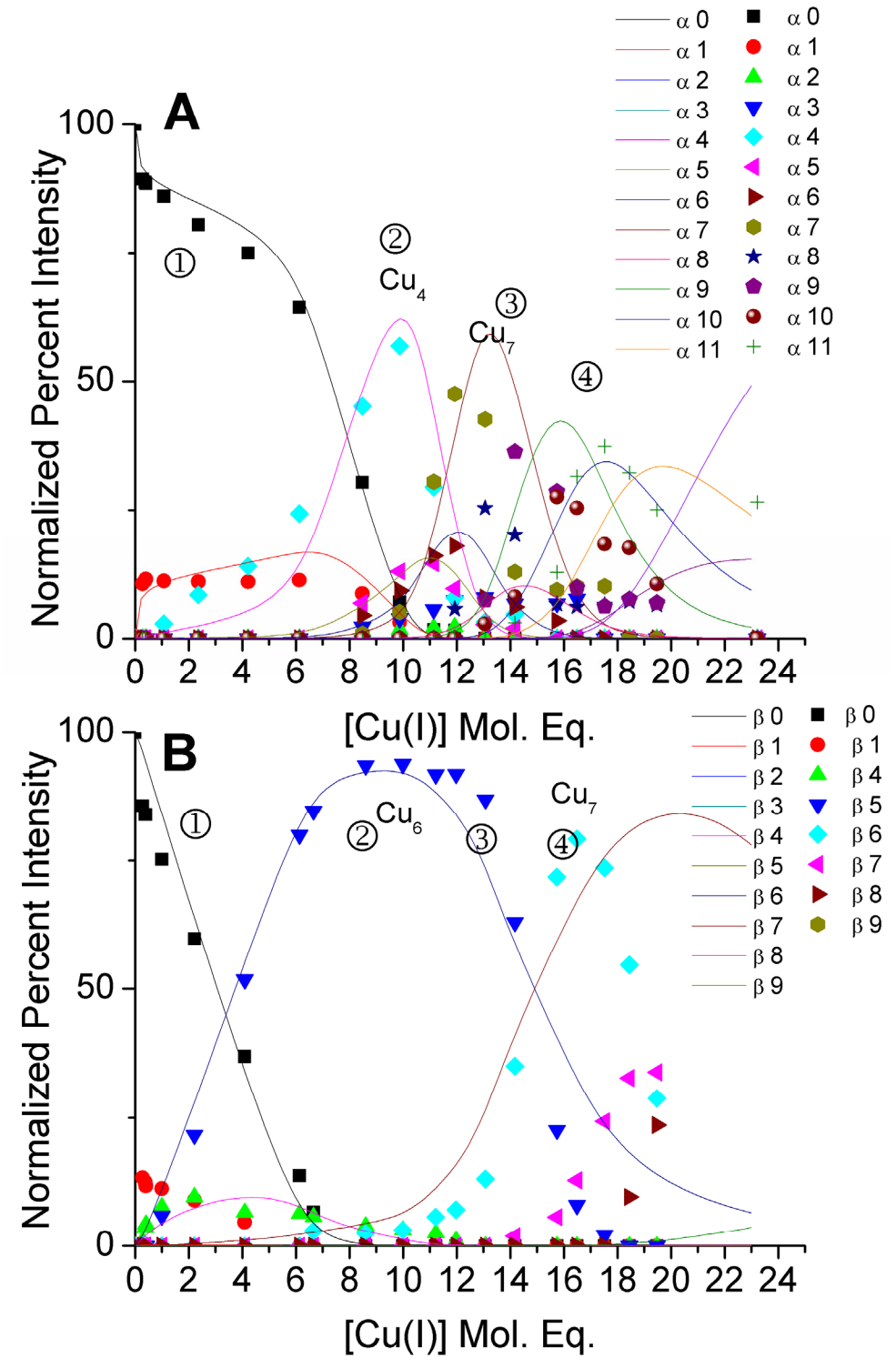


*Fig. S15 Cu(I) speciation following titration of Cu(I) into solution of both apo α (A) and apo β (B) domain fragments with the x- axis showing the amount of Cu(I) bound to both proteins. Speciation from HySS simulation shown as continuous lines and experimental speciation shown as symbols.*

Fig. S14 shows the experimental speciation as points connected by straight lines for clarity with the X-axis being the amount of Cu(I) bound to each respective protein. Fig. S15 shows the experimental (symbols) and HySS simulation for the competition between the α and β domain fragments.

**Competitive binding between the β domain fragment and the full βα MT**

Fig. S16 shows selected deconvoluted ESI-mass spectra from the Cu(I) titration into a solution of 45.6 μM βα MT and 22.6 μM β domain fragment. Despite having a lower concentration of the β domain fragment in solution than the full protein, the signal intensity for the β domain fragment is much higher which indicates a higher ionization efficiency.

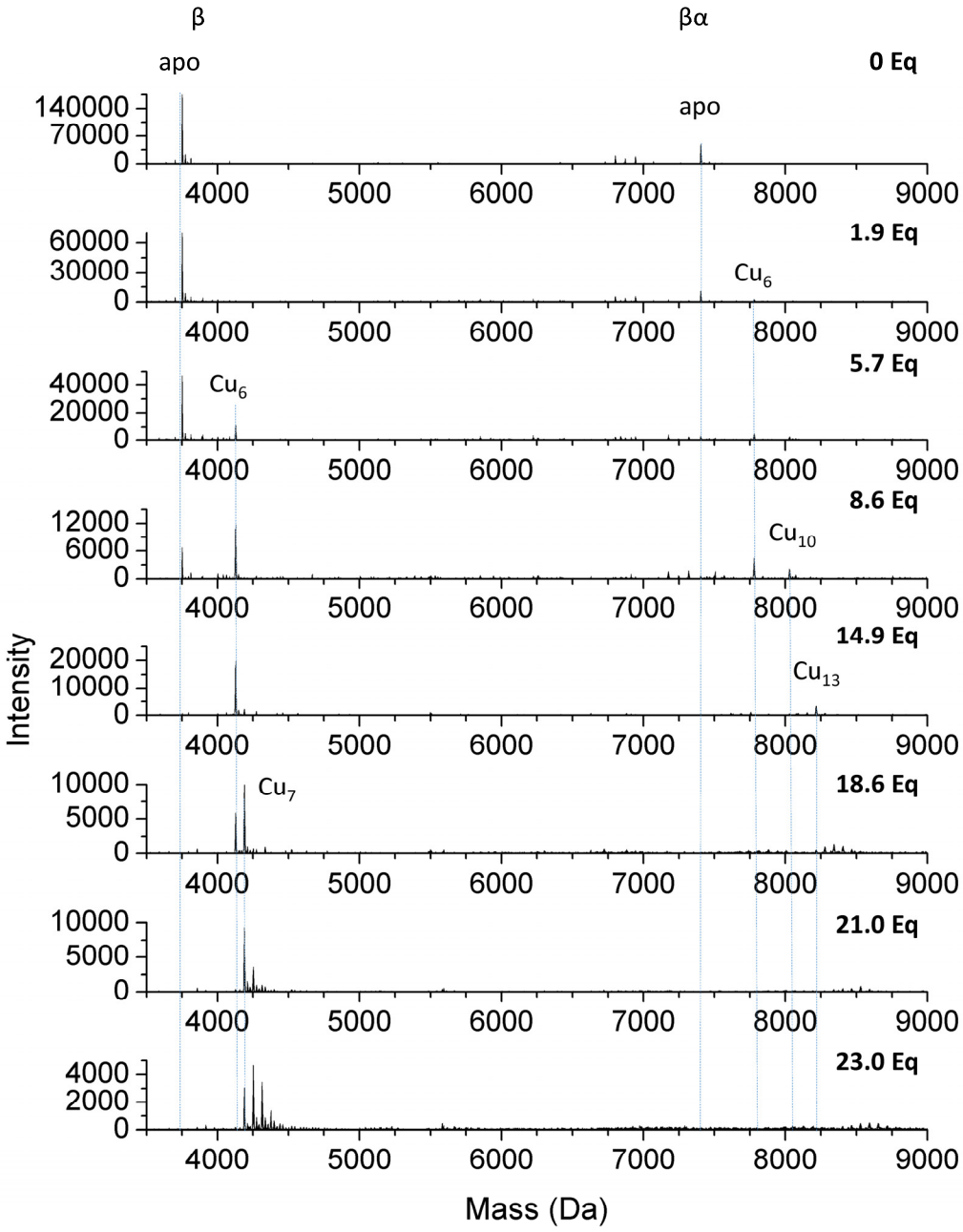


*Fig. S16 Deconvoluted ESI-mass spectral data for the titration of Cu(I) into a mixture of 45.6 μM apo βα and 22.6 μM apo β at pH 7.4 and room temperature.*

Fig. S17 shows the experimental speciation of Cu(I) binding to the solution of βα MT (A) and the isolated β domain (B) with the two speciation graphs separated for clarity. Lines have been drawn through the points, again for clarity. The X-axis has been modified from Fig. 10A, B to only display the Cu(I) bound to each protein. This results in speciation graphs that look very similar to when Cu(I) is titrated into the proteins independent of each other.

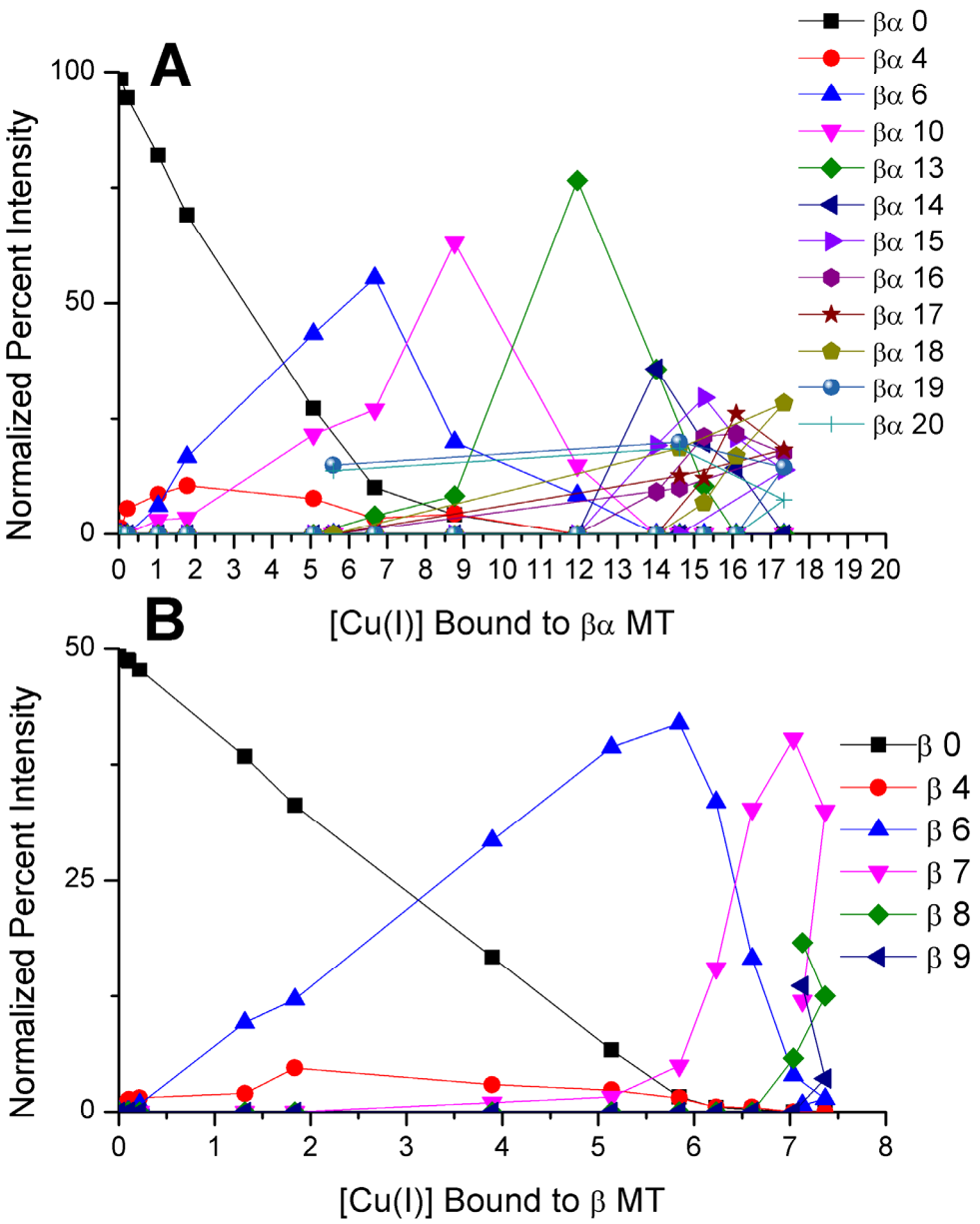


*Fig. S17 Cu(I) speciation following titration of Cu(I) into solution of both apo βα and apo β domain fragments with the x- axis showing the amount of Cu(I) bound to A: βα MT .and B: the β domain fragment.*

**Competitive Binding between α domain fragment and full βα protein**

Equimolar amounts of the α domain fragment and the βα protein were mixed. Cu(I) was titrated into the solution at room temperature and physiological pH. The deconvoluted ESI-mass spectra for various points of the titration are shown in Fig. S18. For clarity, the deconvoluted spectra have been included rather than the charge state data.

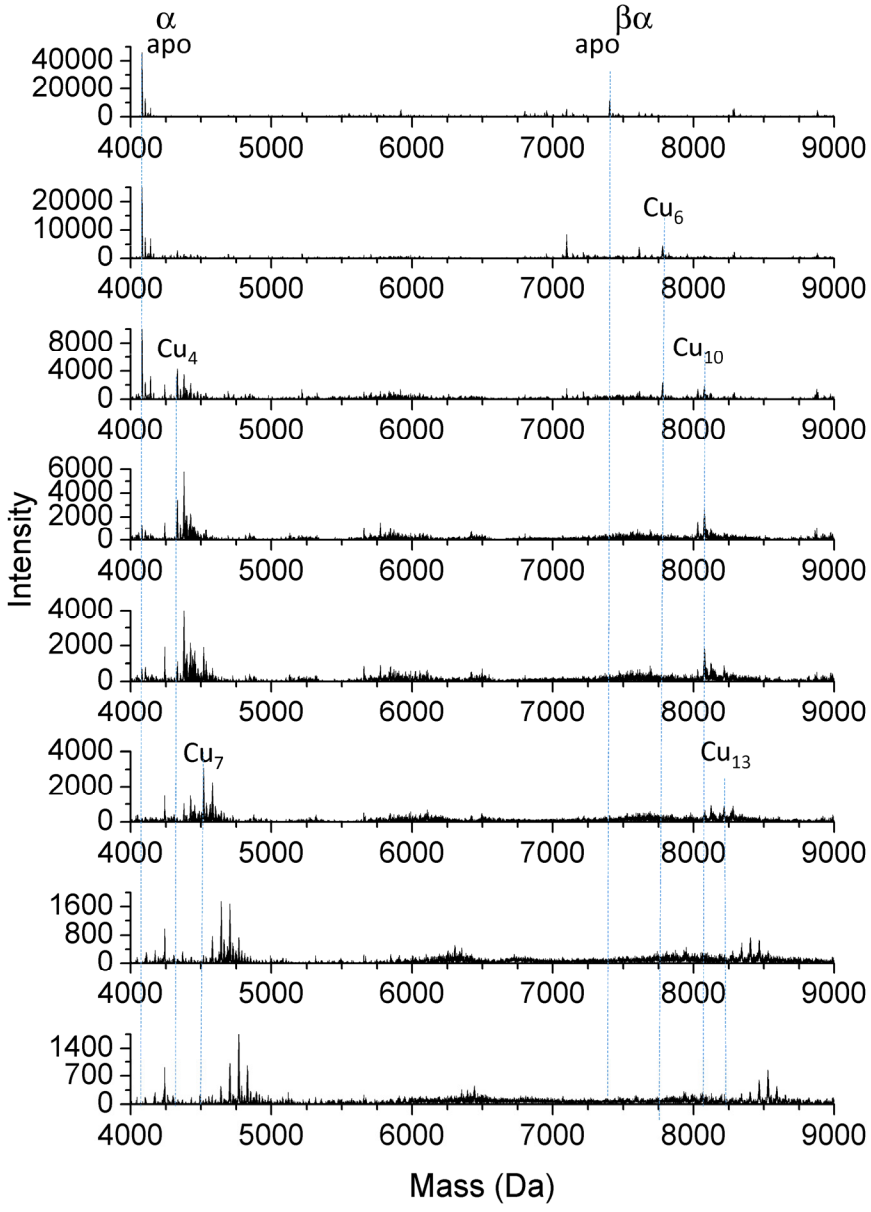


*Fig. S18 Deconvoluted ESI-mass spectral data for the titration of Cu(I) into an equimolar mixture of βα and the α domain fragment at pH 7.4 and room temperature.*

Fig. S19 shows the experimental speciation of Cu(I) binding to the solution of βα MT (A) and the isolated α domain (B) with the two speciation graphs separated for clarity. Lines have been drawn through the points, again for clarity. The X-axis has been modified from Figs. 10D and 10E to only display the Cu(I)

bound to each protein. This results in speciation graphs that look very similar to when Cu(I) is titrated into the proteins independent of each other.

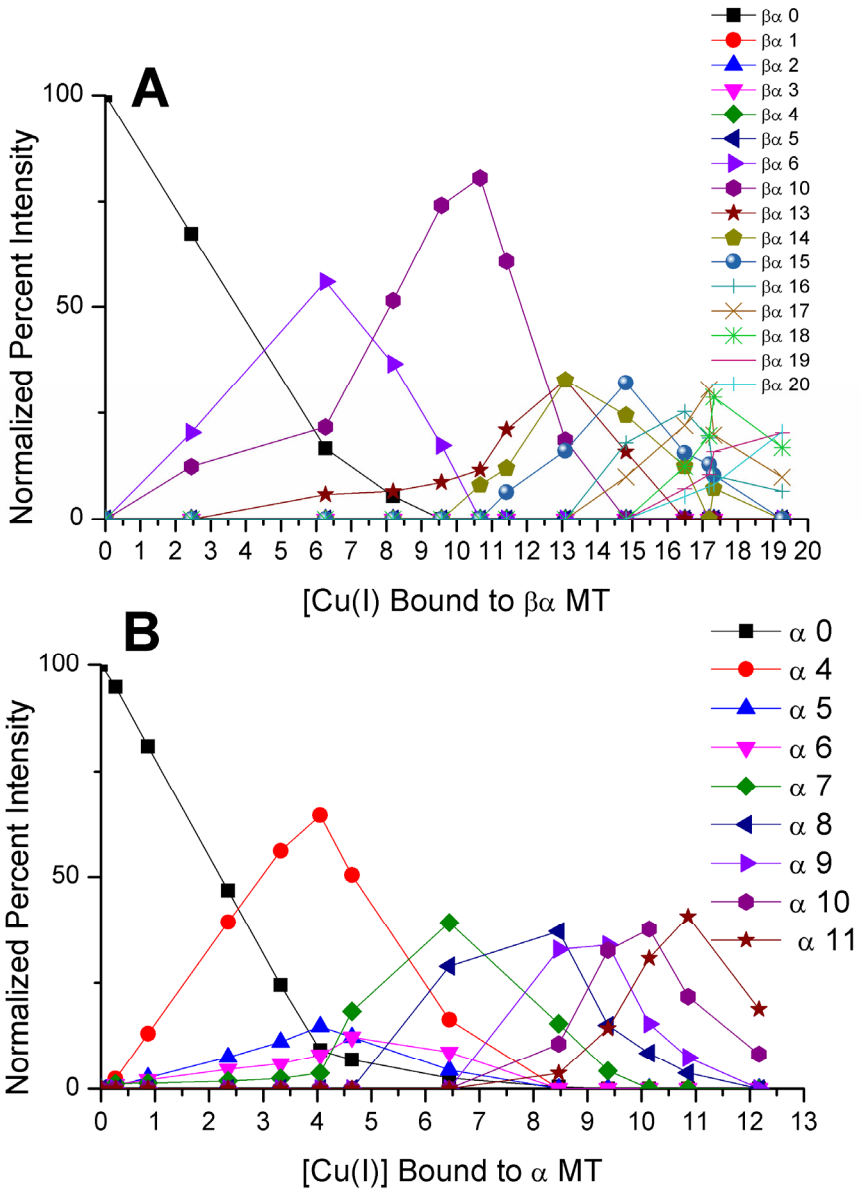


*Fig. S19 Cu(I) speciation following titration of Cu(I) into solution of both apo βα and apo α domain fragments with the x- axis showing the amount of Cu(I) bound to A: βα domain MT. and B: the α domain fragment.*

Fig. S20 shows the HySS speciation (continuous lines) over the experimental speciation (points) for Cu(I) binding to an equimolar solution of βα MT and the α domain fragment. The HySS speciation was used to

generate the relative binding constants for Cu(I) binding to the full βα MT and the isolated α domain.

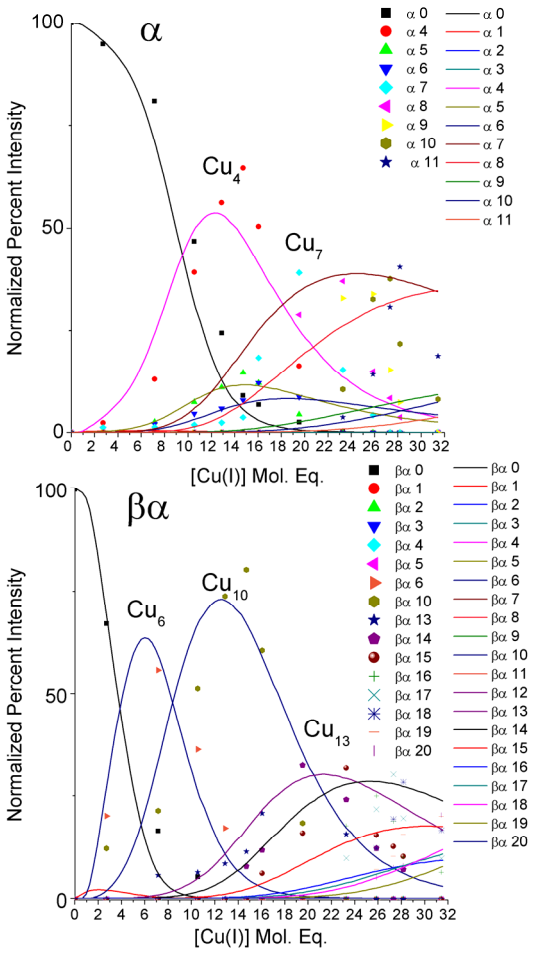


*Fig. S20 Cu(I) speciation following titration of Cu(I) into solution of both apo α (A) and apo βα (B) domain fragments with the x-axis showing the amount of Cu(I) bound to both proteins. Speciation from HySS simulation shown as continuous lines and experimental speciation shown as symbols.*

**Binding constants for Cu(I) binding to βα, β domain fragment, and α domain fragment**

**Table S1: Log $K_F$ binding constants for Cu(I) Binding to βα MT**

| $Cu_n$ βα | Log $K_F$ |
|---|---|
| 1 | 16.8 |
| 2 | 14.4 |
| 3 | 15.2 |
| 4 | 21.8 |
| 5 | 14.1 |
| 6 | 20.7 |
| 7 | 13.2 |

| | |
|---|---|
| 8 | 12.4 |
| 9 | 20.3 |
| 10 | 19.6 |
| 11 | 15.8 |
| 12 | 16.3 |
| 13 | 17.0 |
| 14 | 13.5 |
| 15 | 13.4 |
| 16 | 12.6 |
| 17 | 12 |
| 18 | 12.6 |
| 19 | 11.2 |
| 20 | 6.88 |

**Table S2: Log $K_F$ binding Constants for Cu(I) binding to β domain fragment**

| **$Cu_n$ β domain fragment** | **Log $K_F$** |
|---|---|
| 1 | 15.5 |
| 2 | 14.6 |
| 3 | 15.2 |
| 4 | 18.4 |
| 5 | 15.6 |
| 6 | 17.1 |
| 7 | 14.3 |
| 8 | 12.1 |
| 9 | 10.9 |

**Table S3: Log $K_F$ binding constants for Cu(I) binding to α domain fragment**

| **$Cu_n$ α domain fragment** | **Log $K_F$** |
|---|---|
| 1 | 14.4 |
| 2 | 12.6 |
| 3 | 13.4 |
| 4 | 18.6 |
| 5 | 14.1 |
| 6 | 14.6 |
| 7 | 15.2 |
| 8 | 12.8 |
| 9 | 12.8 |
| 10 | 11.6 |
| 11 | 11.7 |