Metallothioneins: Nature's Sponge for Metals!

Connor S. Durfy, Martin J. Stillman
Stillman Bioinorganic Group, Department of Chemistry
The University of Western Ontario, Canada

Metals in Life[1]

Metals serve many biological purposes in plant, animal and human systems. An example of a vital role of metals is in acting as cofactors in enzymes, most recognizably iron in heme. Typically, the binding of metals to proteins to create metalloproteins can serve one or many structural, enzymatic, homeostatic, and storage functions.

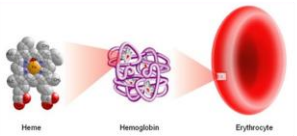

Figure 1: Showing relevance of Iron in heme, and how this relates to larger biological systems

1. Jayawardena, D.; Stillman, M. Expression, Characterization and Metallation Studies of Human Metallothionein Isoform 2a Using Electrospray Ionization Mass Spectrometry. *Western Graduate & Postdoctoral Studies.* **2017**

Copper Ions in Biological Systems[2]

An excess or lack of copper deviating from homeostatic levels in human physiology can lead to ailments such as:

- Wilson's Disease (excess copper in brain and liver)
- Menkes' Disease (excess Cu in small intestines and kidneys resulting in deficiencies)
- Probable connections to neurodegenerative diseases (eg. Alzheimer's disease)

Figure 2: Image showing the symptoms of Wilson's Disease

2. Hudson, E.; Stillman, M. Making a Nano-Necklace: A Study in Metal Binding to Novel Metalloproteins. *Unpublished Thesis.* **2021**

The Biological Importance of Copper[2]

Copper-containing metalloproteins are involved in many different functions in biological systems, including in cellular respiration, the enzymes:

- Cytochrome c oxidase
- NADH dehydrogenase-2

Biological copper is also involved in tissue synthesis, oxygen transport (crustaceans), and other biological processes.

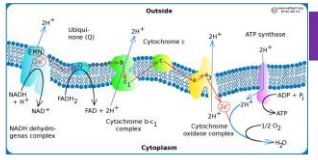

Figure 3: Electron transport chain, showing the importance of copper in biological systems

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Metallothionein (MT)[1]

- Metallothioneins are a class of relatively small and cysteine rich metalloproteins, that have been isolated from both plant and mammalian systems.
- There are four major MT isoforms in mammals:
  - MT1 – in the kidneys
  - MT2 – in the liver
  - MT3 – in the central nervous system
  - MT4 – in squamous epithelial tissue
- Each of the isoforms have similar amino acid sequences.

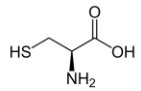

Figure 4: Chemical structure of amino acid L-cystine

- A range of metals induce expression of isoforms 1 and 2
- The sequence in mammalian metallothioneins includes 20 cysteines as shown in the figure
- The isoforms have two identifiable regions of structural significance, beta (9 cysteines) and alpha (11 cysteines)
  - Zn(II), Cd(II), and Cu(I) metallate initially to form metal-thiolate clusters

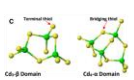

Figure 5: Beta and Alpha domains of MT2 metallated with Cd

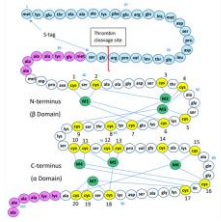

Figure 6: primary amino acid sequence of MT2 with where metals would bind

1. Jayawardena, D.; Stillman, M. Expression, Characterization and Metallation Studies of Human Metallothionein Isoform 2a Using Electrospray Ionization Mass Spectrometry. *Western Graduate & Postdoctoral Studies.* **2017**

Cooperative Metal Binding in MT[3]

At specific ratios of metal to thiol, metallothionein will form unique and predictable clusters, however, isolated cystine metallation precedes this. When metals are added to apo MT we observe predominantly apo and clustered formations of the metallated protein.

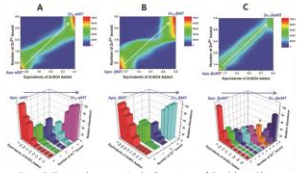

Figure 7: Showing the cooperative binding patterns of the alpha and beta fragments of MT, and the full beta-alpha MT

3. Pinter, T.; Irvine, G.; Stillman, M. Domain Selection in Metallothionein 1A: Affinity-Controlled Mechanisms of Zinc Binding and Cadmium Exchange, *Biochem.* **2015,** 54, 32, 5006-5016

The Importance of MT[2]

In order to maintain homeostatic equilibria, metallothioneins serve as metallo-chaperones for the essential metals zinc and copper. MT may serve in roles such as storage, detoxification, and transport.

Metal free metallothionein is relatively unstructured and the protein is able to accommodate a variety of metals that may be found in the organism, such as: zinc, copper, cadmium, and bismuth.

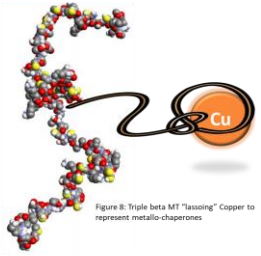

Figure 8: Triple beta MT "lassoing" Copper to represent metallo-chaperones

2. Hudson, E.; Stillman, M. Making a Nano-Necklace: A Study in Metal Binding to Novel Metalloproteins. *Unpublished Thesis.* **2021**

Electrospray Ionisation Mass Spectrometry (ESI-MS)[4]

- Method of analysis capable of determining the mass/charge of all species in the given solution
  - The ESI-MS is able to keep all species intact throughout the process (non-destructive)
- The ESI-MS is able to determine:
  - Structural information – produces charge states of the species
  - Metallation of protein – changes in mass can be seen
  - Oxidation of protein – variations in mass can be seen if the protein has been oxidized


Figure 9: Mechanism of electrospray ionisation

4. Ho, C.; Lam, C.; Chan, M.; et al. Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *Clin. Biochem. Rev.* **2003,** 24(1) 3-12

Methods

Procedural Steps	Laboratory Methods	Instrumental Methods
Cell Growth	- Cells are ordered with pre-inserted plasmids meant to overexpress the protein - Growth occurs in liquid media, additions of IPTG and Cadmium force expression of MT2	- Absorbance of the liquid medium is taken at 600nm to determine the cell density - Centrifuge is used to separate the protein-containing cells from the liquid media
Protein Purification	- Cells are lysed to extract protein, and protein is separated from cell contents and proteases - MT2 is separated from other cell proteins and DNA - The s-tag is cut from the MT2, and then s-tag is removed from the protein solution - Protein is concentrated and frozen for storage	- Cell disruptor is used to pressurize cell medium, thus breaking apart cells allowing for protein extraction - Protein is loaded onto a SPHP cationic exchange column, which is then run using HPLC visualized on the UV-vis to determine when MT2 eludes - S-tag cut is done through an overnight thrombin reaction which targets a specific sequence found on the s-tag - Protein solution is then re-loaded onto the SPHP and run using HPLC to separate s-tag from MT2 - Protein is concentrated using a concentrator until around 20 mL
Sample Clean-up	- Depending on the method of visualization used, the sample is prepared differently, samples are dropped to pH 2 in order to drop out the Cadmium bound to the protein during the growing stage - For ESI-MS there is a buffer exchange from 10mM Tris-HCl (too salty) to 10mM ammonium formate, the solution is also de-salinated - For CD – the protein is diluted to a concentration around 10-20uM - Protein solution is then pH adjusted to the desired pH at which to run the experiment	- Buffer exchange and desalination are done through the use of PD-10 columns, as well as using a centrifuge and spin tubes designed to separate by mass (allowing the salt to fall through and retain the protein)
Sample Visualisation and Experimentation	- Prior to performing the experiment, the concentration of the cleaned protein sample is determined and equivalences of copper are calculated - Both the protein and the copper added are kept under an Argon atmosphere, as the non-metalated protein (Apo) is at high risk of being oxidized once above an acidic pH	- For ESI-MS, protein is pushed through the capillary at a rate of 10uL/min, and m/z of masses 1000-3000Da are recorded - For Steady State Emission, samples are recorded from 570 – 850nm, with excitation at 280nm - For CD, samples are recorded from 220-450nm

Characterising MT2

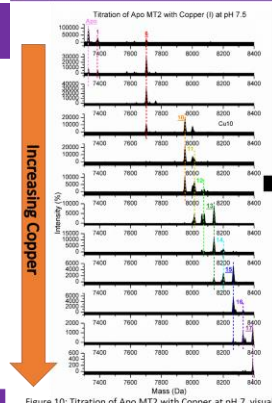

Figure 10: Titration of Apo MT2 with Copper at pH 7, visualized on the ESI-MS

ESI-MS used to determine relative abundance of species in solution, allows for insight into major binding site structures. Visualization of stepwise titrations from Apo to the metallated MT2 provides remarkable detail.

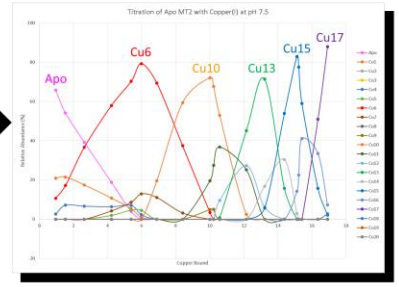

Figure 11: Graph of Apo MT2 Copper titration at pH 7; relative abundance of species against Coppers bound (calculated from ESI-MS data).

Metallated species of MT2 have unique spectral properties, these were also measured on the CD and through steady state emission

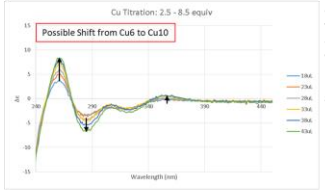

Figure 12: Titration between metallated states as visualised using circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy is able to provide structural information by analysing the chirality of the metal-protein clusters.
Using this method, we can see shifts between metallated states as the clusters' symmetry changes

Steady state emission spectroscopy provides structural information as well. Emission increases when the copper is more shielded from the solvent, meaning it is buried in the protein. Having copper buried protects the cell from uncontrolled reactions occurring.

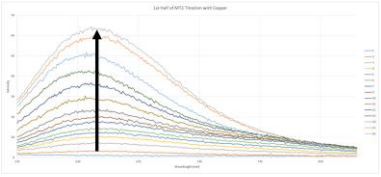
Figure 13: Titration between metallated states as visualised using steady state emission

Acknowledgements

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