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Expression, Characterization and Metallation Studies of Human Metallothionein Isoform 2a Using Electrospray Ionization Mass Spectrometry

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

Maintenance of the homeostasis of zinc (Zn) is very important in regulating bodily functions. There are over 300 Zn dependent enzymes identified, where Zn plays a structural or catalytic role. However, an excess of Zn in a cell is toxic and free Zn ions are tightly controlled. Metallothioneins (MTs) are small cysteine rich proteins, which can bind up to seven Zn ions and act as a Zn reservoir. The MT2a isoform is predominantly found in the liver. My research focused on the overexpression of human MT2a in *Escherichia coli* and the investigation of Zn binding pathways of MT2a *in vitro*. At physiological pH, Zn is terminally bound to the cysteine thiols of MT2a, making bead-like structures (non-cooperative metal binding), while at low pH, Zn formed clusters bridging the terminally bound Zn (cooperative metal binding). These findings draw our attention to investigate how other essential and toxic metals bind to MT2a.

Keywords: Homeostasis, zinc, metallothionein (MT), metallothionein 2a (MT2a), non-cooperative, cooperative, Zn binding pathway

Dedication

I dedicate this thesis to my husband, Asanka. He has always supported me, giving countless help and encouragement through out my academic career.

I also dedicate this thesis to my children, Chathushka, Oneesha and Ethushka. They have missed me a lot when I worked long hours in the lab but were always happy to welcome me when I returned home.

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My foremost thanks go to Dr. Martin Stillman, my supervisor, mentor and friend for the past two years. His patience, support and invaluable advice helped me achieve my research goals. I would like to thank my advisory committee, Dr. Mark Bernards and Dr. Sheila Macfie, for their time and valuable advice, as well as my reader Dr. Sheila Macfie for feedback on my thesis proposal and my thesis. Special thanks go to Dr. Ilka Heinemann in the Department of Biochemistry for her priceless help with metallothionein 2a expression studies. I also would like to thank Dr. Patrick O'Donoghue in the Department of Biochemistry for allowing me to use his equipment and reagents for my research.

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List of Abbreviations

Apo-MT	Apo-metallothionein
<i>p</i> -Bq	<i>para</i> -Benzoquinone
DI water	Deionized water
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ESI-MS	Electrospray ionization mass spectrometry
GSH	Glutathione
Holo-MT	Holo-metallothionein
HPLC	High performance liquid chromatography
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
LB	Lysogeny broth
MOPS	3-(N-Morpholino) propanesulfonic acid
MREs	Metal response elements
mRNA	Messenger RNA
MT	Metallothionein
MT1a	Metallothionein isoform 1a
MT2a	Metallothionein isoform 2a
MTF-1	Metal-responsive transcriptional factor-1
OD	Optical density
RNA	Ribonucleic acid
TCEP	Tris (carboxyethyl) phosphine hydrochloride
Tris-HCl	Tris-hydroxymethyl-aminomethane

UV	Ultra violet
ZIP	Zrt- and Irt-like proteins
ZNT	Zinc transporters

Chapter 1

Introduction

1.1 Essential and toxic metals

All living organisms utilize metals. Some metals such as iron (Fe), zinc (Zn), copper (Cu), magnesium (Mg) and cobalt (Co) are essential and are involved in a myriad of biological functions.¹⁻³ Metals such as lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are toxic to cells.^{1, 2} Zinc is the second-most abundant essential trace element in the human body, at 2.3 g in an average human weighing about 70 kg (Fe is the most abundant; 4 g in an average human), and is required for the function of many proteins.^{4, 5} The concentration of Zn(II) in an eukaryotic cell is very high (10^{-4} - 5×10^{-4} M) compared to other essential metals and the cytosolic free Zn(II) concentration must be controlled within a narrow range (within 10^{-5} M to 10^{-12} M).⁶⁻¹⁰ Metallothionein (MT) exists in the cytoplasm and has the ability to bind essential and non-essential metals in the cell.^{6, 11}

Most toxic metals such as Hg, Cd, Pb, and As are sulfhydryl-reactive metals, and can bind to the sulfhydryl groups of proteins and disrupt their function.¹² For example, Jacobson *et al.*¹³ reported that As(III) causes misfolding and aggregation of Hsp104p-GFP protein in *Saccharomyces cerevisiae*. Mercury and Cd have a high affinity for glutathione (GSH), which is one of the primary antioxidants in the cytosol that protects the body against oxidative and free radical damage.^{14, 15} Another metal binding protein that has been shown to bind to metals using its sulfhydryl groups is MT, which forms metal-thiolate complexes and protects other proteins from dysfunction.¹² In this thesis, I am going to evaluate how Zn binds to MT.

1.2 Biological role of zinc

Zinc contains a filled d orbital (d^{10}); therefore, it is not involved in redox reactions and functions only as a Lewis acid.^{4, 16, 17} This makes Zn a good metal cofactor for enzymes that need redox-stable ions for biological reactions.^{4, 8} Zinc plays a key role in deoxyribonucleic acid (DNA) synthesis, ribonucleic acid (RNA) transcription, cell proliferation and activation, as enzymes such as RNA polymerase, DNA polymerase and transcription factor IIIA are Zn metalloenzymes.¹⁸⁻²⁰

Zinc is also involved in cell signalling.⁹ Zn can be found in glutamate-zinc releasing neurones and neuronal somata that consist of Zn permeable gated channels, as well as all calcium channels that are permeable to Zn.²¹ Kim *et al.*²² revealed that Zn is needed for maturation of mammalian oocytes and the total Zn in an oocyte was higher than the total Fe or Cu. However, excess Zn in biological systems influences misfolding and aggregation of Zn proteins and leads to intermolecular protein-protein interactions.¹⁶

1.2.1 Homeostasis of zinc

The importance of Zn homeostasis was deeply considered in 1958, after reporting an Iranian patient with severe growth retardation, anemia, hypogonadism, hepatosplenomegaly, mental lethargy, geophagia, and rough and dry skin.^{23, 24} These symptoms arise from Zn deficiency. Consumption of only cereal proteins and no meat products caused Zn deficiency for Middle Eastern and people from developing countries.¹⁹ If dietary intake of Zn is reduced, intestinal Zn absorption increases and Zn excretion decreases.²⁵ Although the body has these mechanisms to protect against Zn deficiency, it cannot function properly with chronic deficiency.

It is important to control the efflux and influx of Zn to maintain Zn homeostasis in the cell.¹⁹ Zinc is transported from blood to cells through cell membrane transporters and its concentration is tightly regulated by two families of transport proteins embedded in the cell membrane: Zn transporters (ZnT) that remove Zn from the cell to the blood, and Zrt- and Irt-like proteins (ZIP) that take up Zn from the blood.²⁶⁻²⁸ Maintaining low concentrations of free Zn ions in the cytosol is important for health; concentrations have to be low enough to avoid toxicity, but high enough to provide adequate Zn for cellular function.

1.2.2 Zinc Proteins

In eukaryotic cells, Zn-dependent proteins are mainly found in the cytosol and organelles such as the nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum and secretory vesicles.²⁹ More than 3000 Zn proteins have been identified and more than 300 of these are Zn-containing enzymes, where Zn plays a catalytic or structural role.^{5, 30} Some Zn-containing enzymes, such as carbonic anhydrase and carboxypeptidase, have one Zn atom in their active site.⁵ Alcohol dehydrogenase is another example of a Zn-containing enzyme; in it, Zn plays a structural and catalytic role in the same protein. Each of the two subunits of alcohol dehydrogenase contains a structural Zn atom as well as another Zn atom in their respective catalytic site.³¹ Aspartate transcarbamylase, an enzyme in *Escherichia coli* (*E. coli*), has six Zn atoms, one in each of its six subunits; Zn plays only a structural role in this enzyme.^{5, 32}

Metal-responsive transcriptional factor-1 (MTF-1) consists of six Zn fingers and is responsible for embryonic liver development and metallothionein biosynthesis.³³ Zinc plays a structural role in Zn fingers; these small proteins have Zn in their core and interact

with nucleic acids, proteins and small molecules to control transcription and translation.³⁴ Typical Zn fingers have Cys₂His₂ domains, and the Cys-Cys-His-His motif coordinates to Zn in a tetrahedral geometry.³⁵

There are 15 known ZIP proteins and nine ZnT proteins found in humans.²⁵ Zrt- and Irt-like proteins have eight transmembrane domains and a long histidine-rich loop between domains three and four.²⁹ ZnT however, has six transmembrane domains; the long histidine-rich loop is located between domains four and five.²⁹ Metallothioneins bind free Zn in the cell and act as Zn donors to Zn proteins such as carbonic anhydrase, resulting in low levels of free Zn in the cytosol.²⁶ Bidirectional Zn transfer has been seen between holo-metallothionein to apo-Zn proteins and from holo-Zn proteins to apo-metallothionein, which clearly portrays the zinc donor-acceptor role of MT.³⁶

1.3 Metallothioneins

Metallothioneins are low molecular mass (6-8 kDa), ubiquitous, and cysteine-rich proteins, first isolated from horse kidney cortex in Cd-binding protein studies.³⁷⁻³⁹ Metallothioneins exist in all animal phyla, vascular plants, as well as some prokaryotes.⁴⁰ Detoxification of heavy metals, homeostasis involving essential metals such as Zn and Cu, and protection against oxidative stress in cells system are among the main processes proposed to involve MTs.^{38, 40, 41} These activities arise from the properties of the metallated protein.

1.3.1 Human metallothioneins

There are four isoforms of human MTs (Fig. 1): MT1 and MT2 are the major isoforms of MT, which are found predominantly in the kidneys and the liver, as well as in other tissues; MT3 and MT4 are minor isoforms of human MTs, which are seen only in

unique tissue types such as the brain and the central nervous system, and squamous epithelia, respectively.^{28, 42} A single gene encodes each type of MT2, MT3 and MT4 whereas a set of at least 10 MT1 genes encode the multiple sub types of MT1: MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1J, MT1K, MT1L and MT1X.⁴³⁻⁴⁵

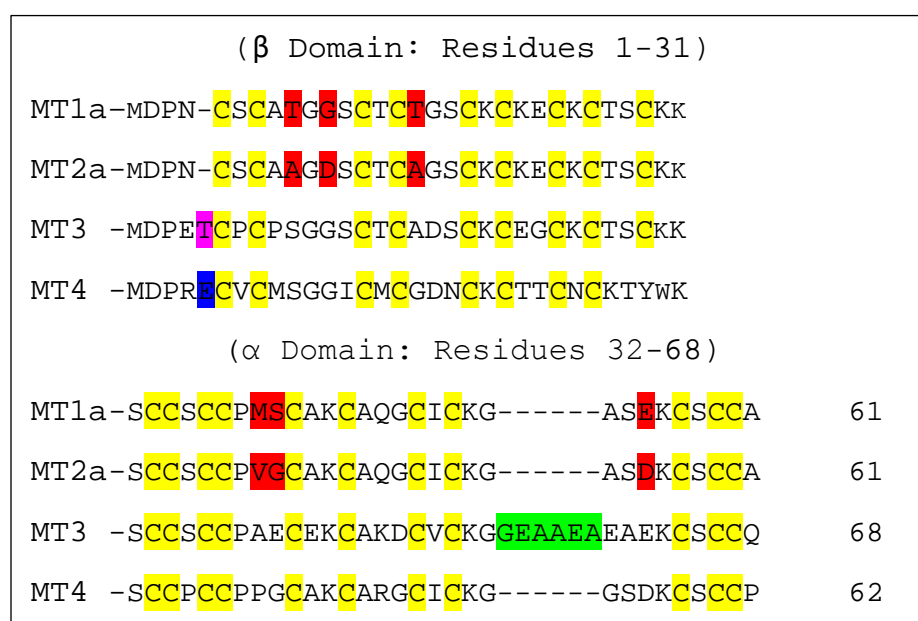


Figure 1. Cysteine alignments of human MT isoform sequences. Both the β domains (Top) and the α domains (Bottom) are shown. The cysteines are highlighted in yellow and different amino acid residues between MT1a and MT2a are highlighted in red. The extra amino acid residues of MT3 and MT4 are highlighted in pink and green, and blue. The figure is modified from Zalewska *et al.*³⁸

MT1 and MT2 are more similar to each other than to MT3, which contains an extra threonine in the β domain and an extra Gly-Glu-Ala-Ala-Glu-Ala motif in the α domain. This hexapeptide motif forms an unordered loop in the C-terminus of MT3.⁴⁶ MT4 has an extra glutamic acid in the β domain. MT4 has eight cysteines in the β domain while all other isoforms have nine cysteines.

Metal binding of MT3 is significantly different from MT1 and MT2 metal binding because it has a lower metal binding affinity and metals bind non-cooperatively.⁴⁶ The

sequence differences of MT3, compared to MT1 and MT2, might be the reason for the metal binding differences.

1.3.2 Structure of human metallothionein

Metallothionein is a monomeric protein with 61-68 amino acid residues of which up to 20 are cysteines.⁴⁷ Metallothionein has two independent metal binding domains: the N-terminal β domain has 9 cysteine residues that coordinate 3 divalent metal ions such as Cd, Zn and Co, and the C-terminal α domain has 11 cysteine residues that coordinate 4 divalent metal ions (Fig. 2).⁴⁸ Metallothioneins bind to monovalent and trivalent metals as well. Monovalent Cu (I) form Cu_6S_{11} and Cu_6S_9 clusters in the α and β domains respectively.⁴⁹ Trivalent As (III) strongly binds to cysteines, forming an As_3S_{11} cluster in the α domain and an As_3S_9 cluster in the β domain.⁵⁰ However, the two domains differ in many ways; for metal binding, Cu preferentially binds to the β domain whereas Cd preferentially binds to the α domain.⁵¹ Moreover, the addition of excess Cd leads to the formation of supermetallated MT; which means that MT can bind up to eight Cd, forming a superdomain.⁴⁷

The thiol groups of the cysteine residues form mercaptide bonds with metals, resulting in metal-thiolate clusters (Fig. 2).⁴⁰ For example, four sulfur atoms bind to Zn(II) in a tetrahedral coordination while three sulfur atoms bind to Cu (I) in a trigonal coordination.^{48, 51} The 20 cysteine residues occur in three different arrangements throughout the protein: Cys-Cys, Cys-X-Cys, and Cys-X-Y-Cys, where X and Y are amino acid residues other than cysteine (Fig. 3).⁴⁰ Disulfide bonds do not form between cysteine residues and there are no aromatic amino acids in the protein.⁴⁸

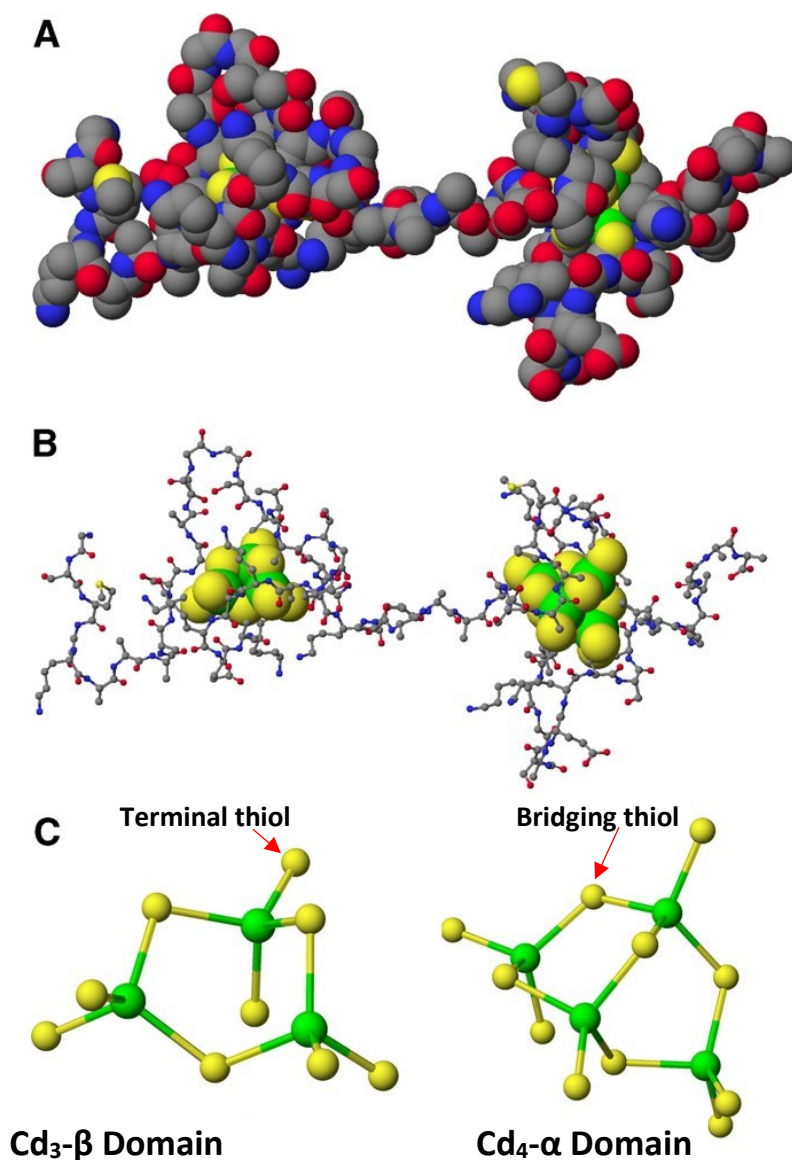


Figure 2. Molecular model structures of human metallothionein 1a. Left side shows the β domain and right side shows the α domain of $\text{Cd}_7\text{-}\beta\alpha\text{MT}$. Entire MT protein is shown in A & B, and the individual domains are shown in C. (A) space filling (B) ball and stick with space filling (C) ball and stick of $\text{Cd}_3\text{-}\beta$ and $\text{Cd}_4\text{-}\alpha$ domains showing the Cd-thiolate cluster formation of MT (one cysteine (yellow) bound to more than one Cd (green) formed clusters). The terminal thiols and the bridging thiols of cysteines are indicated. The figure is adapted from Sutherland & Stillman.⁵²

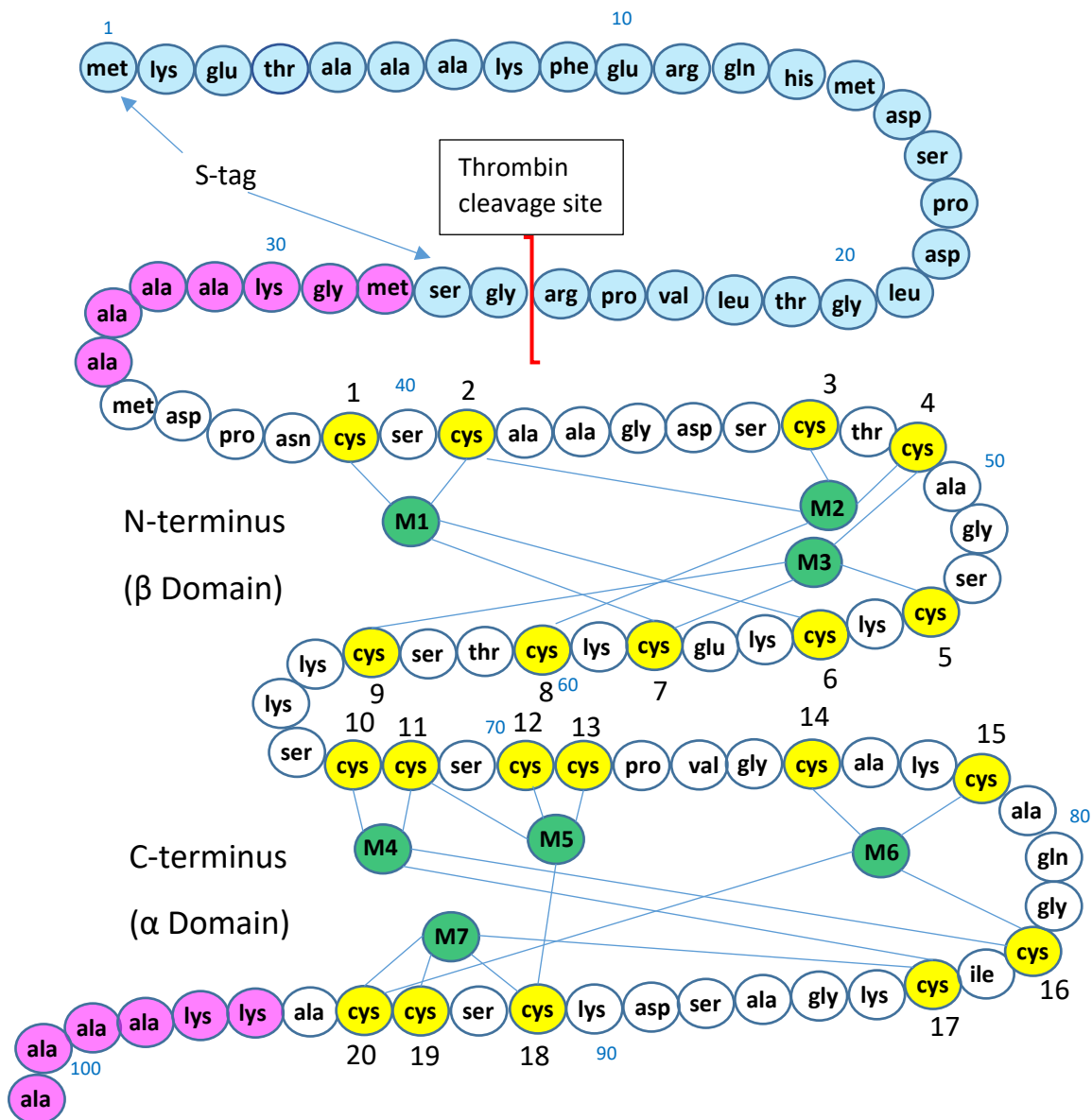


Figure 3. The sequence of human metallothionein 2a with S-tag (blue). Seven divalent metals (green) bind to 20 cysteines (yellow). The cysteines are numbered 1-20. Modifications to the N-terminus (MGKAAAA) and the C-terminus (KKAAAA) are shown in pink. The thrombin cleavage site is indicated.

1.3.3 The origin and biosynthesis of human metallothionein

Metallothionein can be found in the cytoplasm, nucleus, mitochondria and lysosome.^{51, 53} Metallothioneins function both intra and extracellularly.⁵¹ For example, MTs can be found in extracellular fluids such as plasma, urine, pancreatic fluid, amniotic fluid, bile and milk.^{38, 51, 54} Protecting the DNA from oxidative damage or supplying Zn(II) to Zn enzymes and transcription factors are the suggested roles of MT in the nucleus.⁵³

Because MT1 and MT2 are expressed in many cell types in various organs, cell signaling molecules like cytokines, and hormones like glucocorticoids, as well as a variety of stress conditions such as reactive oxygen species, and metal ions induce their biosynthesis but there is no evidence of biosynthesis of MT3 or MT4 from these inducers.⁵⁵⁻⁵⁸ Moreover, MT3 is abundant in non-Zn-containing neurones such as pyramidal neurones.⁵⁹

Transcription of the MT gene is activated by MTF-1, by injection of a metal load including Cd, Zn, Cu, Hg, and bismuth (Bi) to the body.^{33, 51} MTF-1 is a Zn finger protein, consisting of six Zn fingers, and binds to metal response elements (MREs) with a core consensus sequence TGCRNC in the promotor of MT genes.^{33, 60, 61} Inhibition of MT synthesis occurs at the transcriptional and translational level in the cell, by inhibitors such as cycloheximide, actinomycin D, and puromycin.⁵¹

1.3.4 Zinc-metallothionein vs cadmium-metallothionein

Human Zn-metallothionein (Zn-MT) and Cd-metallothionein (Cd-MT) exhibit similar physical and chemical properties, but the biological roles of the two proteins are vastly different.⁴⁸ Zn-MT is synthesized predominantly in the intestine and the liver to regulate the homeostasis of Zn, while Cd-MT forms to protect against Cd toxicity by

storing Cd in the liver, and later in the proximal tubular cells of the kidney cortex after detoxification.^{62, 63} Cherian⁶² found that rats injected with Zn-MT did not show any toxic effect because Zn-MT transferred the Zn(II) to other Zn proteins. In addition, in order to protect highly Cd-sensitive organelles like nuclei and mitochondria from Cd toxicity, MT initially sequesters Cd in the cytosol of hepatocytes in the liver.⁶⁴

Even though we can induce Cd₇-MT by supplying more Cd(II) to animals, native MT proteins consist of Cd₅Zn₂-MT; four Cd(II) bind to the α domain making a stable cluster, and one Cd(II) and two Zn(II) bind to the β domain making a less stable cluster.⁵⁶ The two Zn atoms in the β domain are exchangeable.⁵ However, once MT is saturated with Zn(II) or Cd(II), MT is not susceptible to degradation by proteases.⁵⁶

1.3.5 Metallothioneins in human diseases

Some diseases show elevated or decreased levels of all or some isoforms of MT. Significant increases of MT have been seen in patients with pancreatitis due to an increase in free radical activity, and a decrease in free radical scavengers, such as superoxide dismutase, catalase, glutathione peroxidase, vitamin E and C.⁵³ Patients with Alzheimer's disease show a significant decrease of MT3 in their brain, the major isoform found in the brain and central nervous system.⁶⁵ Patients with neurological diseases such as Alzheimer's disease, Pick's disease, Creutzfeldt-Jacob disease, amyotrophic lateral sclerosis, and multiple sclerosis show elevated levels of MT1 and MT2 in their brain.⁶⁵

There is a direct relationship between cancer cell proliferation and overexpression of MT in the following cancers: squamous cell carcinoma of the esophagus, thyroid cancer, uterine cervical squamous tumor, ovarian cancer, breast cancer, and nasopharyngeal

cancer.^{44, 66, 67} Jin *et al.*⁶⁶ have shown that MT2 mRNA levels are higher than MT1 in breast cancer tissues. However, MT1b is absent in breast cancer tissues.⁴⁴

1.4 Metallation and metal binding affinity of metallothionein

Metal-dependent protein folding is characterized by the coordination of one or more metal ions by the protein to give its fully functional conformation.¹¹ One good example of metal-dependent protein folding is the Cd-dependent folding of MT (Fig. 4). *In vitro*, the binding affinity of metal ions to mammalian MTs shows the following order: Zn(II) < Pb(II) < Cd(II) < Cu(I), Ag(I), Hg(II), Bi(III).³⁸ Metallothioneins show a higher affinity for Cd(II) than Zn(II), such that even Cd scarcely dispersed in the environment will result in Cd accumulation in MTs.⁴⁰ The binding constant of Cd₇-MT2a is $3.2 \times 10^{17} \text{ M}^{-1}$ at pH 7.4 whereas the binding constant of Zn₇-MT2a is $3.2 \times 10^{13} \text{ M}^{-1}$ at pH 7.4.³⁶ Although toxic, the 10,000 times greater binding affinity of Cd(II) for MT compared with Zn(II) means that ingested Cd(II) will displace Zn(II) in Zn-MT.⁴⁰

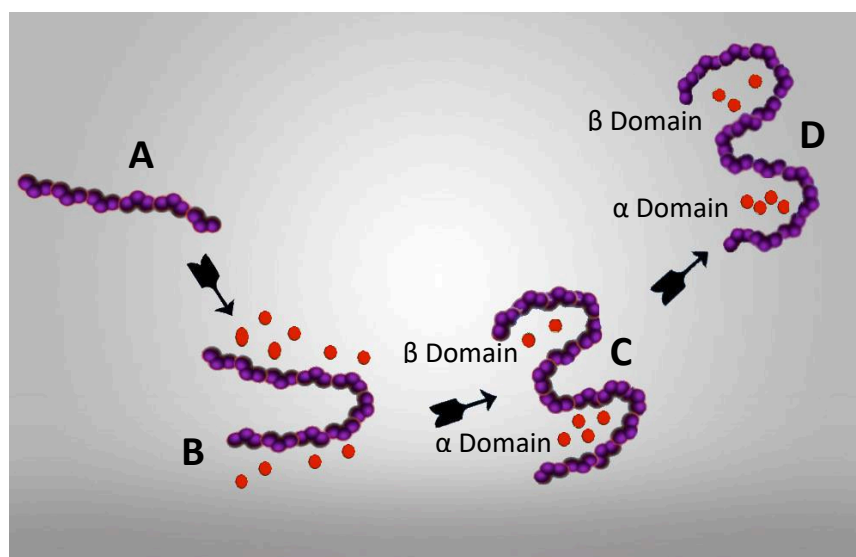


Figure 4. Metal-dependent protein folding of metallothioneins *in vitro*. (B) Red circles represent Cd ions, which induce the synthesis of MT. (C) Metallation starts in the α domain binding up to four Cd, then the β domain. (D) Seven Cd ions bind to MT; the α domain consists of four Cd and the β domain consists of three Cd.

1.4.1 Cooperativity

Cooperativity can be seen in many biological systems and can be explained in many ways. Cooperativity describes how the binding of the first ligand to a receptor influences the binding of subsequent ligands.⁶⁸ Metal binding can be divided into three groups; positive cooperativity, negative cooperativity, and non-cooperativity.⁶⁸ In positive cooperative metal binding, the equilibrium constant (K) for each subsequent ligand increases with the addition of a metal, which facilitates the next metal ion binding.¹¹ A well-known example of positive cooperativity is the binding of oxygen molecules to hemoglobin.^{69, 70} Binding of the first oxygen molecule increases the affinity of the protein for a second oxygen molecule, then the third and fourth molecules as well.⁶⁸ In nature, only deoxygenated hemoglobin or oxygenated hemoglobin can be observed because intermediates will not have resulted in extreme positive cooperativity.^{71, 72} When the binding of one ligand interferes with the binding of the subsequent ligands to the protein, the binding constant is lowered and this is called negative cooperativity.^{71, 73} The binding of insulin to its receptor shows negative cooperativity.⁷³⁻⁷⁵ If subsequent binding events have identical K values, it is called non-cooperative binding.⁶⁸

Allosteric cooperativity occurs when the interaction of a ligand with a protein changes the conformation of the protein.⁷⁶ The phenomenon of **i**³ cooperativity is derived from allosteric cooperativity and displays the following characteristics: binding of a ligand induces the conformation of the multi-subunit protein, the conformational changes are **i**namolecular, and the binding sites are **i**dentical to one another.⁷⁶

1.4.2 Proposed metallation pathways for metallothionein

There are two proposed metallation pathways for human metallothioneins: cooperative and non-cooperative.⁷⁷ The cooperative metal binding pathway of MT is not quite the same as the cooperative protein-ligand interactions discussed in section 1.4.1. For MT, the metals bind to terminal thiols and then form stable metal clusters through bridging thiols; this phenomenon is the cooperative metal binding pathway of MT (Fig. 5).^{47, 77} Terminally bound intermediates are less dominant in this pathway.^{47, 77} Vasak & Kagi⁷⁸ have shown that the first four Co ions bind to terminal thiols of rabbit liver metallothionein 1 in a tetrahedral geometry prior to the formation of Co-thiolate clusters. When divalent metals bind to the seven metal binding sites on MT, metal binding starts at the α domain in a cooperative manner, forming a stable cluster, whereas the β domain follows the cooperative manner but forming a less stable cluster.^{56, 79, 80} However, demetallation follows the reverse order of metallation; the β domain releases its metals prior to the α domain.^{56, 81}

Non-cooperativity occurs when one metallation event does not facilitate the subsequent metallation events.⁵² Each metallation event happens independently at each domain.⁵² In the non-cooperative metal binding pathway of MT, metals bind to terminal thiols and make bead-like structures (Fig. 5).^{47, 77, 82} The four metal clusters are less dominant in this pathway; the intermediates can be seen and metal speciation shows a normal distribution.⁷⁷

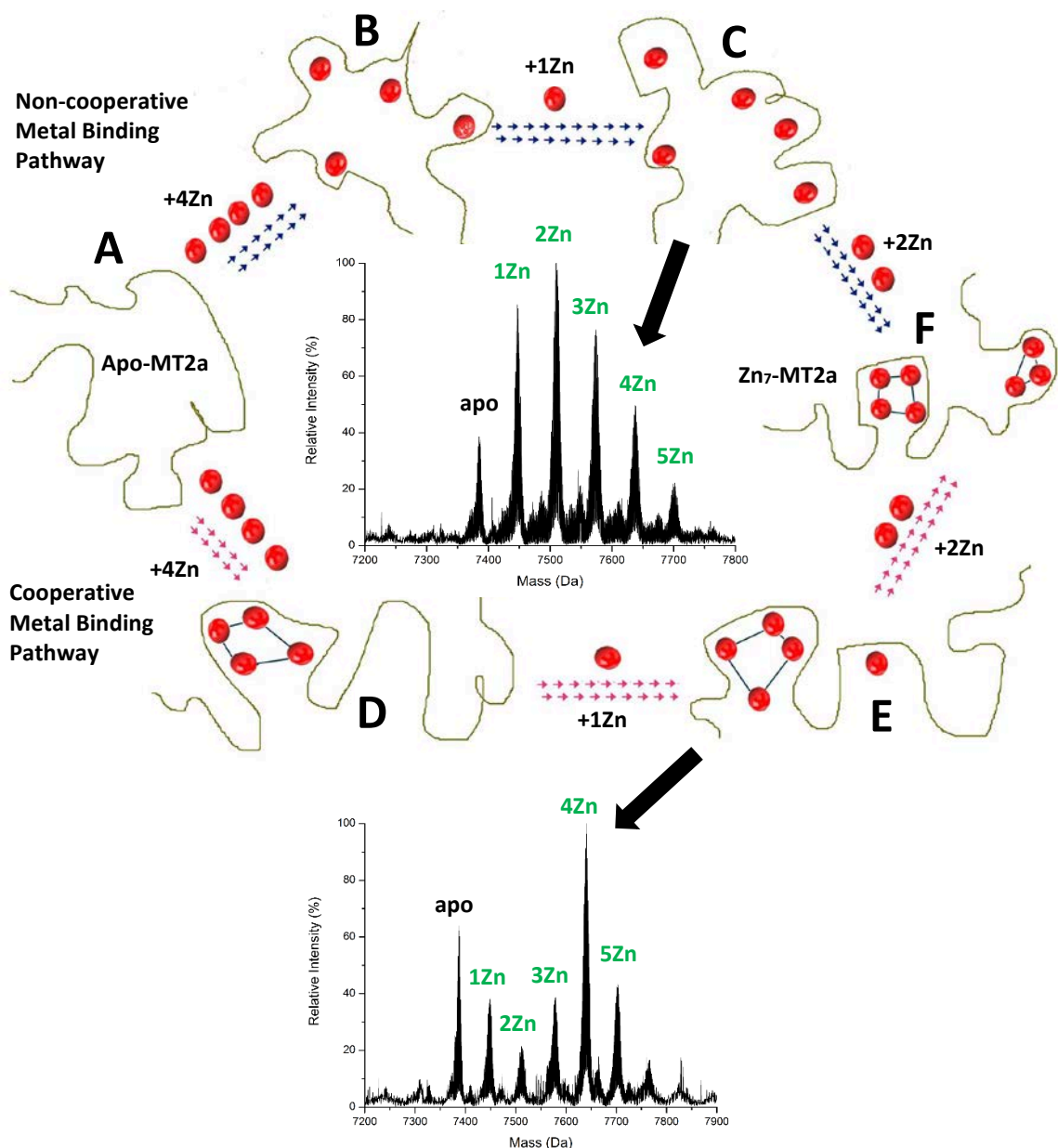


Figure 5. Two proposed metallation pathways of MT for divalent metals; Zn(II) binding to MT2a is used as an example. The cooperative (cluster formation) pathway is shown following pink arrows (D & E) and non-cooperative (beads formation) pathway is shown following blue arrows (B & C). In the non-cooperative pathway, 20 cysteine residues are terminally bound to five metals, and the 6th and 7th metal bind to form two stable clusters. In the cooperative pathway, four metals bind to 11 cysteine residues forming a stable M₄S₁₁ cluster (D) and then three metals bind to nine cysteine residues forming two stable clusters (F). The intermediates (Zn₁-MT2, Zn₂-MT2 and Zn₃-MT2) are dominant in the spectrum is shown at top (beads formation) and clusters (Zn₄-MT2) are dominant in the spectrum is shown at bottom (cluster formation). The figure is modified from Irvine *et al.*⁷⁷

1.4.3 pH and metallothionein

The intrinsic pKa of cysteine is 8.6 and positively charged residues will lower the pKa, whereas negatively charged residues will raise the pKa of the cysteine.⁸³ Below the pKa of cysteine, metals compete with protons.^{48, 84} Due to the high proton content at low pH, metals compete with protons to bind to MTs and form bridges between terminal thiols resulting in metal-thiolate stable clusters.⁷⁷ At high pH, competition of metals and protons to MT thiols are less intense due to lower proton content in the environment and metals still bind to terminal thiols of MTs, but cluster formation is not dominant.⁷⁷ Therefore, protons influence the metallation of MTs *in vitro*. This is similar to the binding of oxygen to hemoglobin in the human, where the binding affinity of oxygen is influenced by protons, organic phosphates, chlorides and carbon dioxide.⁸⁵

1.5 Hypothesis

Because metal binding reactions are dependent on the exact amino acid sequence of the protein, I hypothesize that metallation will differ between the liver MT2a and the kidney MT1a. I think that non-cysteine amino acids in the MT2a and MT1a influence the metal binding properties of Zn.

I predict that at low pH, MT2a will show a cooperative metal binding pathway whereas at physiological pH, MT2a will show a non-cooperative metal binding pathway. Low pH destabilizes the formation of individual bead-like structures and favours the formation of clusters.

1.5.1 Objectives of this research

One goal of this research was to overexpress human MT2a in *E. coli*, which is a very difficult protein to express in *E. coli* and the Stillman group has been trying to express this protein for several years.

My second objective was to determine the pathways of Zn(II) binding to MT2a with changing pH, monitored using Electrospray Ionization Mass Spectrometry (ESI-MS).

Chapter 2

Materials and Methods

2.1 Modifications to the MT2a sequence

Expression studies were conducted using the MT2a sequence previously generated by the Stillman lab. (The MT2a sequence, with the S-tag without modifications to the N and the C-termini, is shown in Figure 3). A series of test expressions were carried out to identify the optimal isopropyl- β -D-1-thiogalactopyranoside (IPTG) concentration (0.1-5 mM) and optimal temperature (16°C, 20°C, 30°C and 37°C) for maximum yield of MT2a. The pET 29a plasmid with the *MT2a* gene was transformed to the following cell lines: BL21 (DE3), BL21 C+, Rosetta 2, BLR pLysS, Nova Blue (DE3), Rosettablue pLysS, Tuner pLysS, BLR (DE3), B834 pLysS and BL 21 pLysS. The *MT2a* gene was cloned into the pGEX-6p2 vector with the GST tag. Low yields were obtained for the expression of MT2a and the yield could not be optimized using common techniques due to the instability of the protein. Therefore, further details of these expression studies were not included in this thesis.

An *MT2a* gene within the pET29a plasmid in BL21 (DE3) cells was poorly overexpressed and MT2a yield was too low to carry out experiments using ESI-MS. A new MT2a sequence was designed by adding amino acids to the N-terminus (MGKAAAA) and the C-terminus (KKAAAA) (Fig. 3). The DNA sequence of MT2a was synthesized and cloned into the pET29a plasmid using restriction enzymes, NcoI and HindIII completed by GENEWIZ (South San Francisco, CA 94080, USA). The resulting protein with the S-tag is shown below. The S-tag is highlighted in blue, extra amino acid residues added to the N

and C termini are highlighted in red and the native human MT2a protein sequence is in bold font:

**MKETAAAKFERQHMDSPDLGTLVPRGSMGKAAAAMPNCSCAAGDSCTCAG
SCKCKECKCTSCKKSCCSCPVGCAKCAQGCICKGASDKCSCCAKAAAA**

2.1.1 Solutions and agar plates preparation

The 50 mg/mL kanamycin antibiotic was prepared by adding 0.5 g of kanamycin powder into 10 mL of deionized water (DI water). The 1 M IPTG solution was prepared by dissolving 2.38 g of IPTG solid in 10 mL of DI water. Both solutions were filtered using a 0.2 μ m syringe filter and stored at -20°C. 1 M cadmium sulfate (CdSO₄) was prepared by dissolving 5.13 g of CdSO₄ solid in 20 mL DI water and stored at room temperature.

Lysogeny broth (LB) agar (Miller) was prepared by dissolving 10 g of LB agar in 250 mL water, then the solution was autoclaved at 121°C for 15 minutes. After the liquid cooled to room temperature, 250 μ L of the kanamycin solution was added and the mixture quickly poured into 10 sterile Petri dishes, and left at room temperature for 30 minutes (The final concentration of kanamycin in the agar plates was 50 μ g/mL). After the agar in the Petri dish hardened, it was stored at 4°C. Regular LB agar plates were prepared following the same method without the addition of kanamycin and stored at 4°C.

Liquid LB media (Miller) for bacterial pre-cultures was prepared by dissolving 2.5 g LB into 100 mL DI water. Growth media for bacteria was prepared by dissolving 25 g of LB media in 1 L of DI water. A 4L of LB media was prepared. All liquid LB media were autoclaved at 121°C for 15 minutes and stored at room temperature.

2.1.2 Making competent cells

Competent cells, TOP20 and BL21 (DE3), were prepared by following method: the TFB1 buffer was made by mixing 30 mM potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$) (1.472 g), 10 mM calcium chloride (CaCl_2) (0.555 g), 50 mM manganese chloride (MnCl_2) (4.948 g), 100 mM rubidium chloride (RbCl_2) (6.046 g) and 15% glycerol in 425 mL DI water. The pH was adjusted to 5.8 and 75 mL of glycerol was added to the solution. The TFB2 buffer was made by mixing 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (1.046 g), 75 mM CaCl_2 (4.162 g), 10 mM RbCl_2 (0.604 g), and 15% glycerol in 425 mL DI water. The pH was adjusted to 6.5 and 75 mL of glycerol was added to the solution. Both buffers were stored at 4°C.

Both TOP10 and BL21 (DE3) cells were plated on agar plates (without antibiotics) and incubated over night at 37°C. The next morning, one colony of each cell types was transferred into 25 mL LB medium and the cells were grown at 37°C using a shaker (Excella E24 incubator shaker series, New Brunswick Scientific) at 175 RPM, until an optical density (OD) of 0.6 (600 nm) was reached. Cells were harvested by centrifugation (Beckman Coulter, Avanti J-26 XPI) at 6000 RPM for 15 minutes at 4°C. After removing the supernatant, 10 mL of TFB1 buffer was added to the cell pellets and incubated on ice for 5 minutes. The cell mixture was then centrifuged again for 5 minutes at 3000 RPM at 4°C and the supernatant was removed. A 400 μL aliquot of the TFB2 buffer was added to each tube and incubated on ice for 30 minutes. The competent cells were divided into 40 μL aliquots and stored at -80°C.

2.1.3 Transformation of pET29a plasmid with *MT2a* gene into *E. coli*

Lyophilized pET29a plasmids including the *MT2a* gene were mixed with 40 μL of autoclaved DI water. A 2 μL aliquot of plasmid mix was added into 40 μL competent TOP10 *E. coli* cells and subjected to a 42°C heat shock for 45 seconds. Immediately after the heat shock, the cells were cooled on ice for 2 minutes. Afterwards, 1 mL of preheated LB medium was added to the TOP10 cells and incubated at 37°C for 60 minutes. Transformed TOP10 cells were plated on LB agar plate with kanamycin (50 $\mu\text{g}/\text{mL}$), and incubated overnight at 37°C. The next morning, one colony was transferred to 10 mL LB medium with 10 μL kanamycin (50 mg/mL) and grown on a shaker at 175 RPM at 37°C until it reached the target OD₆₀₀. The plasmids were extracted using a Presto™ mini plasmid kit (Geneaid) as per the manufacturer's instructions. A 2 μL aliquot of extracted plasmids were transformed into BL21(DE3) competent cells using the above protocol. BL21 (DE3) cells were plated to an LB agar plate with kanamycin (50 $\mu\text{g}/\text{mL}$) and incubated overnight at 37°C. One colony was transferred to 10 mL LB media with 10 μL of kanamycin (50 mg/mL) and grown on a shaker at 175 RPM at 37°C until it reached the target OD₆₀₀. Glycerol stocks were made to store the cells in a -80°C freezer. A 650 μL aliquot of cell culture was transferred into autoclaved 1.5 mL Eppendorf tube and mixed with 350 μL 80% autoclaved glycerol.

2.1.4 Overexpression of metallothionein 2a in *E. coli*

To make the pre-cultures, 100 mL of autoclaved LB medium was transferred into each of 4 Falcon tubes after adding 100 μL of kanamycin (50 mg/mL). The four pre-cultures were inoculated by 1 μL of glycerol stock, which included the *E. coli* with pET 29a plasmid carrying the *MT2a* gene, to each tube. The four pre-cultures were incubated

overnight at 37°C in the shaker at 100 RPM. The next morning, the pre-cultures were transferred into four autoclaved flasks with 1 L LB medium, 1000 µL kanamycin (50 mg/mL) and 50 µL 1 M CdSO₄. All four cultures were incubated in the shaker (175 RPM) at 37°C. BL21 (DE3) cells with the *MT2a* gene were grown until they reached the target OD₆₀₀. It took approximately four hours to reach the OD₆₀₀. Then, 600 µL of 1 M IPTG was added to each flask to induce protein expression. After half an hour of induction, 150 µL of 1 M CdSO₄ was added to the LB media to aid the proper folding and stabilization of MT2a and the cells were left to grow for three hours. Cells were harvested by centrifugation (Beckman Coulter) at 6000 RPM for 15 minutes at 4°C. After removing the supernatant, cell pellets were stored at -80°C.

2.1.5 Purification and cleavage of S-tag

For purification, 10 mM Tris-hydroxymethyl-aminomethane (Tris-HCl) and 10 mM Tris-HCl with 100 mM sodium chloride (NaCl) buffers were used. To make Tris-HCl buffer, 1.211 g of Tris-HCl was dissolved in 1 L of DI water and the pH was adjusted to 6.1 using hydrochloric acid (HCl). To make 10 mM Tris-HCl with 100 mM NaCl, 1.211 g of Tris-HCl and 5.844 g of NaCl were dissolved in 1 L of DI water and the pH was adjusted to 6.1.

The cell pellets were thawed in 10 mM Tris-HCl buffer at pH 6.1 and lysed using a cell disrupter (Constant Systems, UK) shot at 20, 24, and 26 kpsi for two times each at 4°C. The cell lysates were kept on ice to minimize protease activity. The cellular debris was removed by centrifugation (Beckman Coulter) at 13000 RPM for one hour at 4°C and the supernatant was transferred onto a cation exchange column (Hi TrapTM SP HP - GE Healthcare) with a total volume of 10 mL (two 5 mL columns were attached together). To

remove loosely bound protein and cellular debris, the columns were washed for approximately two hours with 10 mM Tris buffer at pH 6.1. The gradient of salt was increased slowly using 100 mM NaCl with 10 mM Tris at pH 6.1 and the MT2a was eluted using high performance liquid chromatography (HPLC). An ultra violet (UV)-visible absorption spectroscopy was used to detect the MT2a. Varian, Cary 50 Bio, software was used to detect the cadmium-thiolate ligand-to-metal charge transfer band at 250 nm to isolate MT2a. The diluted MT2a was concentrated to 30 mL by membrane pressure filtration with Ultracel 5 kDa ultrafiltration discs (Millipore).

Metallothionein 2a was made with an epitope tag (S-tag) at the N-terminus for solubility and identification purposes and was cleaved off prior to the experiments using a Thrombin Clean-Cleave Kit (Sigma-Aldrich). Preparation and regeneration of Thrombin beads were done as per the manufacturers' instructions. The thrombin enzyme cleaves the peptide bond between Arg and Gly, recognizing the consensus sequence of Leu-Val-Pro-Arg-Gly-Ser. The thrombin beads were mixed with MT2a and kept at 4°C for 12 hours. Then, the protein mixture was diluted 10-fold to reduce the salt concentration and transferred to the HP SP cation exchange columns. The columns were washed for 30 minutes with 10 mM Tris-HCl buffer at pH 6.1. The S-tag was eluted at low salt concentration because it was not bound to the columns as strongly as MT2a. The MT2a protein was eluted by increasing the salt gradient as mentioned above and then concentrated to approximately 100 μ M. Metallothionein 2a was divided into 2.5 mL aliquots and stored at -20°C. The concentrated, purified MT2a was desalted by size exclusion chromatography and characterized by ESI-MS (Fig. 6).

2.1.6 Desalting and demetallation of Metallothionein 2a

A 10 mM ammonium formate (NH_4HCO_2) buffer was made by adding 0.631 g formic acid, ammonium salt solid to 1 L of DI water and the pH was adjusted to 7.4 using formic acid. The frozen MT2a aliquots were stored at -20°C were thawed in an evacuator. A PD-10 desalting column (GE Healthcare) was used to desalt MT2a. First, the desalting column was washed three times with deionized water at pH 1.9. Then the fourth time, 10 drops of formic acid were added to the column. 2.5 mL of MT2a was then added to the column. To elute MT2a, 3.5 mL of deionized water at pH 1.9 was added to the column and the eluate was collected into an Amicon Ultra-4 centrifugal filter tube (Millipore). Then 10 drops of formic acid were added to the centrifugal tube to demetallate the MT2a. The centrifugal tube with 3.5 mL of protein solution was centrifuged for half an hour at 3900 RPM using CS6 centrifuge (Beckman Coulter) at 4°C . The tube was refilled with argon saturated 10 mM ammonium formate buffer at 7.4 pH and then centrifuged again for 30 minutes. The last step was repeated for twice. Desalted, demetallated MT2a was transferred to acid washed vials and capped loosely. The vial was kept in the evacuator to evacuate oxygen, then argon saturated prior to experimentation.

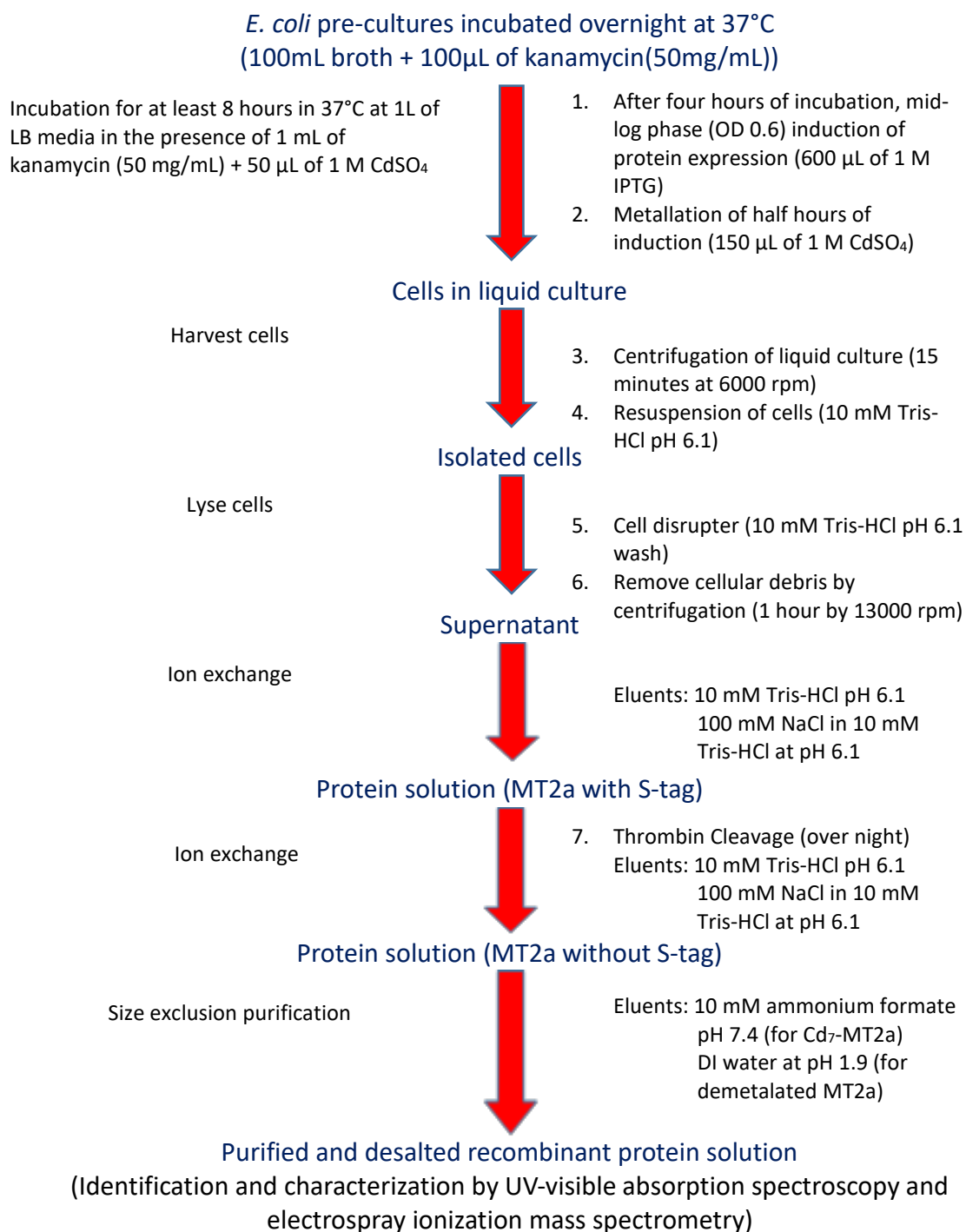


Figure 6. A flow chart of MT2a preparation; showing the overexpression, purification, cleavage of the S-tag and demetallation of recombinant human metallothionein 2a.

2.2 Electrospray ionization mass spectrometry measurements

All ESI mass spectra were acquired using a Bruker Micro-TOF II electrospray ionization mass spectrometer (Bruker Daltonics, Toronto, ON) in the positive ion mode. A 10 mM NH_4HCO_2 buffer was used to prepare the protein solution and it served as the electrospray carrier solvent in the positive ion mode. The mass spectrometer was calibrated using sodium iodide (NaI). Settings: scan = 500-4000 m/z; rolling average = 2; nebulizer = 2 bar; dry gas = 180°C @ 6.0 L/min; capillary = 4000 v; end plate offset = -500 V; capillary exit = 175 V; Skimmer 1 = 30.0 V; Skimmer 2 = 23.5 V; Hexapole RF = 800 V. The flow rate of the protein solution was 10 $\mu\text{L}/\text{min}$. ESI-MS spectra were recorded for two minutes and the Maximum Entropy application of the Bruker Compass Data Analysis software package was used to deconvolute the spectra.

2.3 Modifying metallothionein 2a with *p*-benzoquinone

p-Benzoquinone (Bq) is a cysteine modifier used to quantify the free cysteines in a protein.⁸⁶ Cysteine is one of the most reactive amino acids in the protein and is very prone to post-translational modifications.⁸⁶ *p*-Benzoquinone covalently binds to free cysteines in MT2a, which causes a mass shift detectable using ESI-MS.

2.3.1 Benzoquinone experiment

A 100 mM Bq solution was prepared (0.1081 g of Bq mixed with 3 mL methanol and then added to 7 mL deionized water) and covered with aluminium (Al) foil to avoid reaction with light. Argon was bubbled into the Bq solution to displace any oxygen in the solution. *p*-Benzoquinone was then added stepwise to the desalted 134 μM apo-MT2a and the ESI-MS spectrum was recorded for two minutes. The amount of Bq in the protein solution was increased until the amount of apo-MT2 decreased and the Bq saturated protein

dominated. A 10 μL Tris (carboxyethyl) phosphine hydrochloride (TCEP) was added to the protein-Bq solution to reduce disulfide bonds. TCEP stock solution was made by dissolving six crystals of TCEP in 10 mM NH_4HCO_2 buffer.

2.4 Zinc titration of metallothionein 2a at low pH

A 10 mL stock solution of 50 mM zinc acetate ($\text{Zn}(\text{O}_2\text{CCH}_3)_2$) was prepared by mixing 0.1097 g of zinc acetate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) with 10 mL DI water and filtered using a 0.2 μm syringe filter. The first experiment at low pH was conducted to find the Zn binding pH of MT2a. A 312 μM solution of apo-MT2a was prepared and the ESI-MS spectrum was recorded for two minutes at pH 4.1. Then 7 μL of 50 mM $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ was added to the MT2a solution and the spectrum was recorded for two minutes. The pH was changed to 4.6 and the spectrum was recorded for two minutes.

The second experiment at low pH was conducted to check whether all the Zn(II) in the solution binds to apo-MT2a at low pH. More than 2.5 molar equivalents of Zn(II) (0.9 μmol) was added to 312 μM apo-MT2a at pH 4.7 and recorded on the ESI-MS for two minutes. The pH was increased to 5.2 and the spectrum was recorded for two minutes.

2.4.1 Zinc titration of metallothionein 2a at high pH

The first experiment was conducted to determine how Zn(II) binds to apo-MT2a between pH 6 and 7. The ESI-mass spectrum for 312 μM apo-MT2a was recorded for two minutes prior to the experiment. A 2 μL aliquot of 50 mM $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ was added into the 312 μM apo-MT2a solution and the ESI mass spectrum was recorded for two minutes. Zinc acetate was added stepwise to apo-MT2a until the Zn(II) in the protein solution reached 2.0 molar equivalents. Each metallation step was recorded for two minutes using ESI-MS.

After the molar equivalents of Zn(II) reached 2.5 in the solution, the pH of the solution was increased to 7.8 to conduct the second experiment. An ESI mass spectrum was recorded for the solution at 7.8 then the pH was changed to 7.4, 7.1, 6.1, 5.1, 4.9, and 4.7. An ESI mass spectrum was recorded at each pH until salt adducts from the $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ solution became the dominant species in the solution.

2.5 Donating zinc from holo-metallothionein 2a to apo-metallothionein 2a

To prepare Zn-MT2a, 0.9 μmol of $\text{Zn}(\text{O}_2\text{CCH}_3)_2$ was added to 312 μM apo-MT2a at pH 7.4 and a mass spectrum of the holo-MT2a was recorded for two minutes. A 300 μL aliquot of 134 μM apo-MT2a was added to the solution and left for two minutes for the reaction to complete. Each ESI mass spectrum was recorded for two minutes.

Chapter 3

Results

3.1 Human Metallothionein 2a with S-tag bound to seven cadmiums and apo-metallothionein 2a with S-tag

Recombinant human MT2a was made with an S-tag. Cadmium was used to induce metal dependent protein folding of MT2a and seven Cd ions were incorporated to the protein (Fig. 7A). The mass difference of 59 Da indicates the carboxymethylation of one cysteine in MT2a (Fig. 7A & B). Acidification with formic acid removed Cd(II) from Cd₇-MT2a, resulting in apo-MT2a with an S-tag (Fig. 7B).

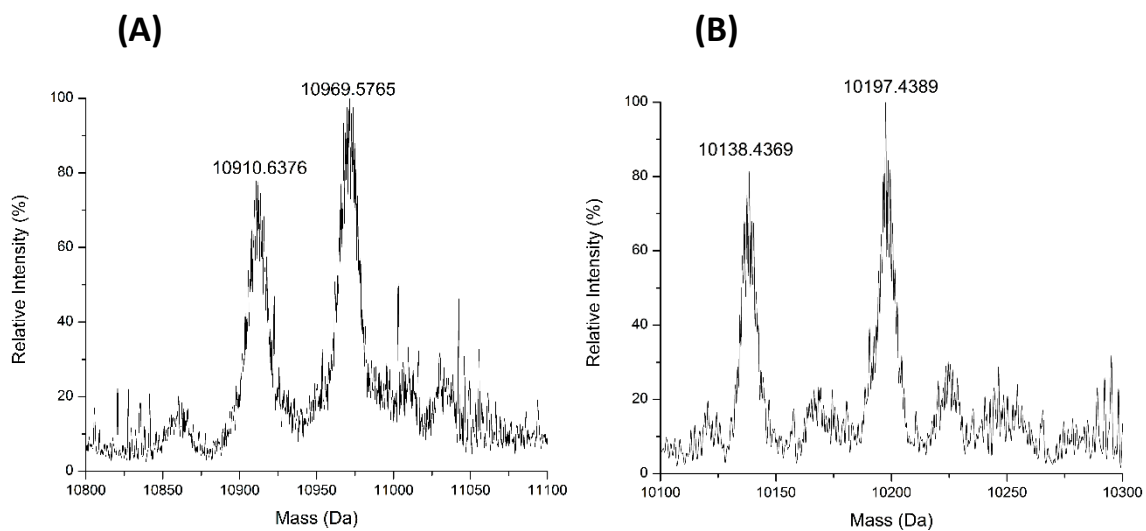


Figure 7. Representative ESI mass spectra of Cd₇-MT2a with S-tag and apo-MT2a with S-tag. (A) The mass of MT2a with S-tag and seven Cd (10910 m/z) and mass of carboxymethylated MT2a with S-tag and seven Cd (10969 m/z). (B) Acidified MT2a and carboxymethylated MT2a showing the mass difference after removal of seven Cd.

3.1.1 Human Metallothionein 2a bound to seven cadmiums and apo-metallothionein 2a followed by removal of S-tag

The S-tag on the N-terminus of recombinant human MT2a was removed before conducting the Zn binding studies (Fig. 8A). Thrombin enzyme cleaved the S-tag between the Arg and Gly residues, keeping the Gly and Ser residues with the MT2a. Acidification with formic acid removed the seven Cd(II) that were incorporated into MT2a. This resulted in the formation of apo-MT2a and a protein mass change (Fig. 8B).

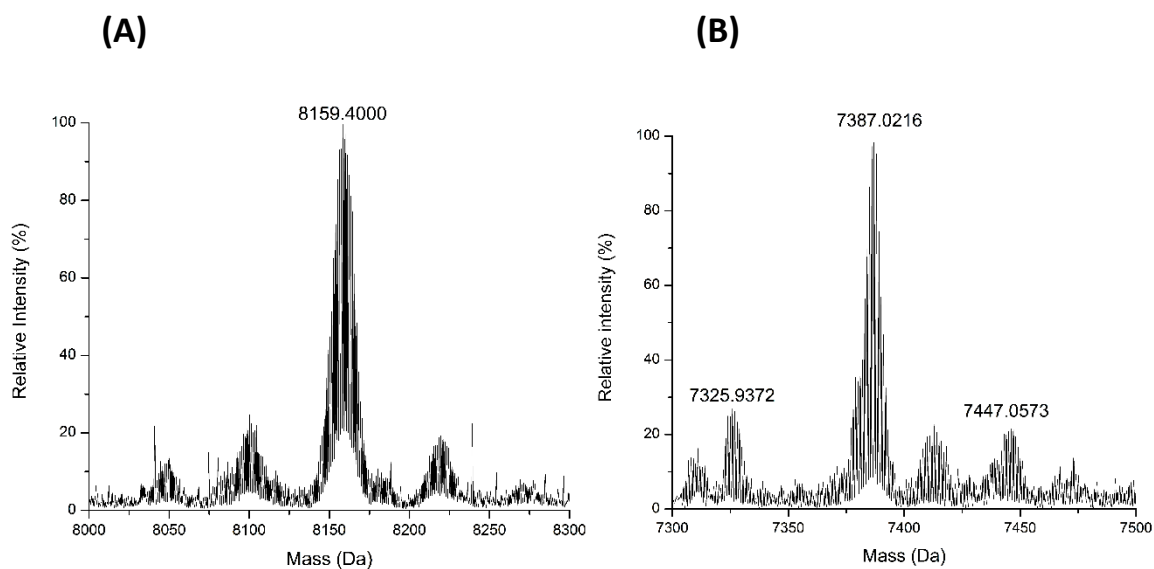


Figure 8. Representative ESI mass spectra of carboxymethylated Cd₇-MT2a and apo-MT2a. (A) The mass of carboxymethylated MT2a with seven Cd followed by cleavage of S-tag (8159 m/z). (B) Acidified carboxymethylated MT2a (7387 m/z) showing the mass difference after removal of seven Cd.

3.2 Quantification of free cysteines in metallothionein 2a

To better understand how many of the 20 cysteines in MT2a are free thiols, studies with the sequential addition of Bq were conducted (Fig. 9). Only 19 Bq were incorporated into apo-MT2a, which indicates that one out of the 20 cysteines was post-translationally modified. Addition of TCEP did not make any difference to the spectrum. If there were disulfide bonds between cysteines (Cys-S-S-Cys), TCEP would reduce the sulfurs, breaking the disulfide bond. There were no disulfide bonds between cysteines in apo-MT2a and the reason why the 20th Bq molecule was not incorporated to the MT2a was likely because a carboxymethyl group was incorporated into one of the cysteines in MT2a (Fig. 7).

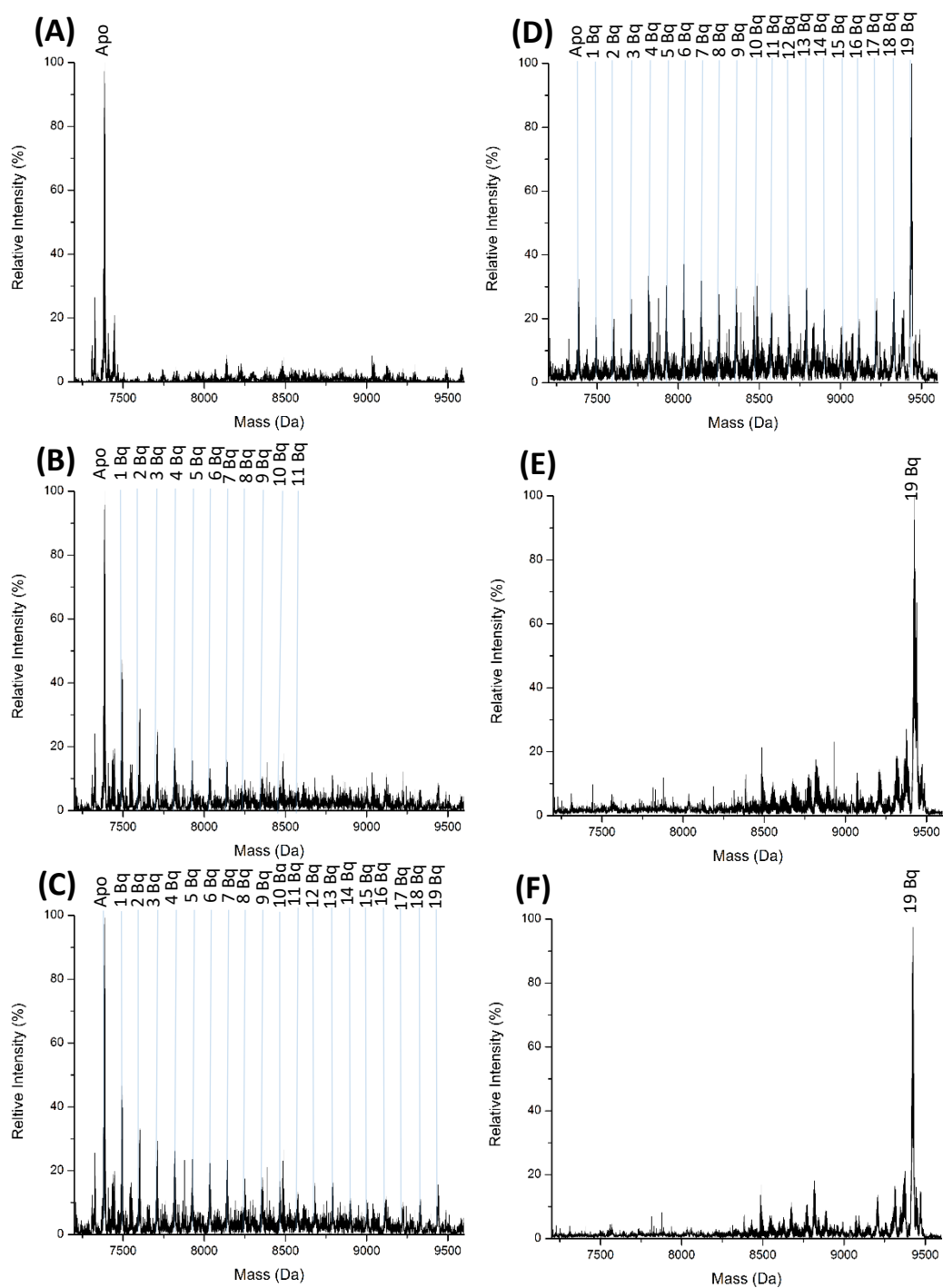


Figure 9. Representative ESI mass spectra for apo-MT2a titrated with *p*-benzoquinone at pH 4.5. (A)-(F) represent of 0 μmol, 0.25 μmol, 0.5 μmol, 1 μmol, 1.2 μmol, and 1.2 μmol of Bq in total, respectively. (F) consists of 10 μL TCEP in addition to Bq.

3.3 Finding the metallation pH of zinc at acidic condition

Zinc was not incorporated into apo-MT2a under acidic conditions, showing that there is no Zn (II) binding at pH 4.1 (Fig. 10B). Zinc metallation started at pH 4.6. Figure 10C shows that Zn binding follows the cooperative metal binding pathway, where the cluster formation was dominant.

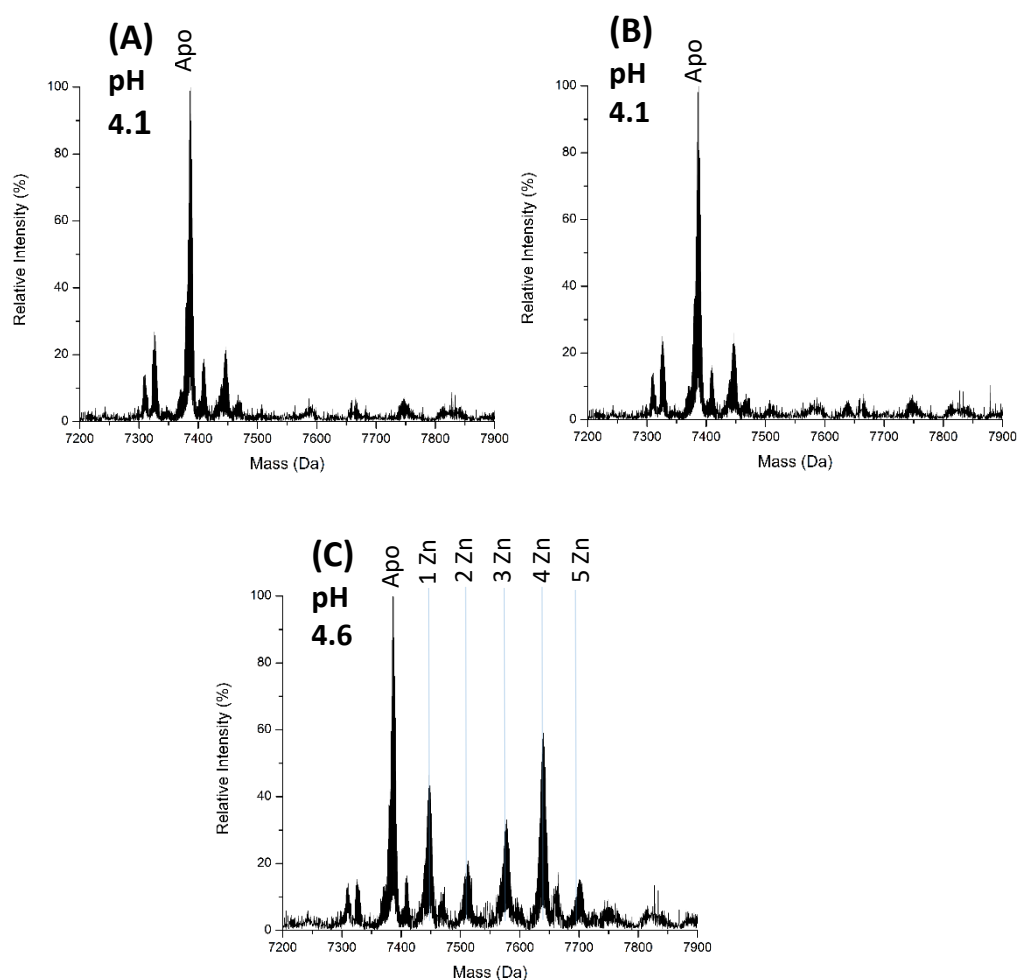


Figure 10. Representative ESI mass spectra for apo-MT2 titrated with zinc acetate at pH 4.1 and 4.6. (A) 312 μ M apo-MT2a with no Zn. (B) 7 μ L of 50 mM zinc acetate added to apo-MT2a at pH 4.1. (C) The pH was raised to 4.6 with no change in the amount of Zn(II) in the solution.

3.3.1 Apo-metallothionein 2a binds relatively low zinc percent at low pH

At low pH (e.g., 4.7), only some of the Zn(II) in the solution bound to apo-MT2a due to the acidity of the solution (Fig. 11A). When the pH of the solution was increased to 5.2, the incorporation of Zn(II) into apo-MT2a increased and no apo-MT2a was left in the solution (Fig. 11B). The amount of Apo-MT2a, Zn₁-MT2a, Zn₂-MT2a and Zn₃-MT2a were reduced and Zn₅-MT2a, Zn₆-MT2a and Zn₇-MT2a were increased when the pH shifted to 5.2. Zn₄-MT2a did not show much difference (Fig. 11A & B). This shows the cooperative nature of Zn binding.

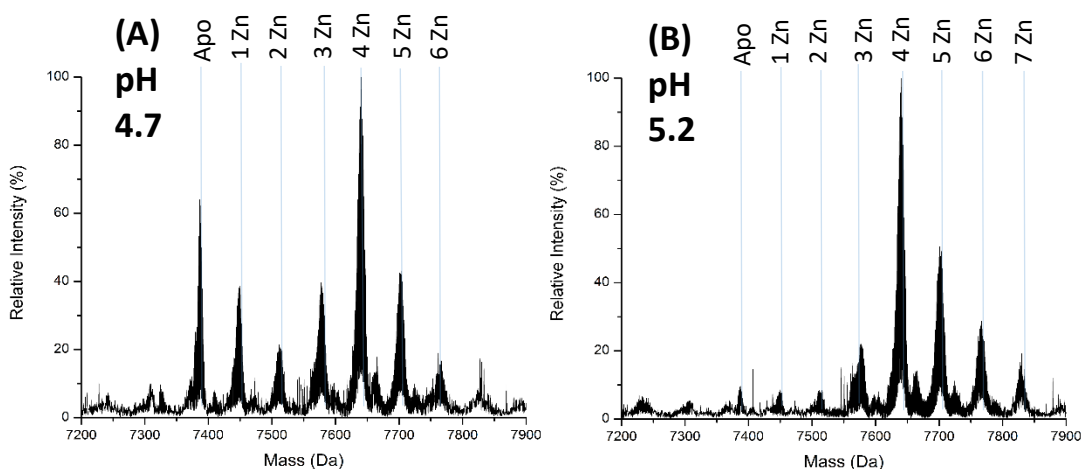


Figure 11. Representative ESI mass spectra for Zn-MT2 at pH 4.7 and 5.2. (A) A 0.9 μmol of zinc acetate in the solution. (B) The pH was raised to 5.2 with no change in the amount of Zn(II) in the solution.

3.3.2 Sequential addition of zinc to apo-metallothionein 2a between pH 6 and 7

Decreasing amounts of apo-MT2a were observed when the molar equivalents of Zn(II) were increased stepwise between pH 6 and 7 (Fig. 12). Zinc binding to apo-MT2a showed that non-cooperative binding pathway dominated around pH 6 and 7. Intermediates of Zn₁-MT2a, Zn₂-MT2a, Zn₃-MT2a and Zn₅-MT2a were dominant during the titration. Zinc speciation showed a normal distribution around Zn₂-MT2a, suggestive of the bead-like structures; proposed for the non-cooperative metal binding pathway (Fig. 12D & E).

The molar equivalents of Zn(II) were calculated using the ESI mass spectral data from Figure 12 (Fig. 13). A decrease in apo-MT2a and an increase in Zn-MT2a was observed with the sequential addition of 0 μmol , 0.1 μmol , 0.15 μmol , 0.05 μmol , and 0.075 μmol of 50 mM Zn(O₂CCH₃)₂ respectively (0.35 μmol total Zn(O₂CCH₃)₂ in the solution).

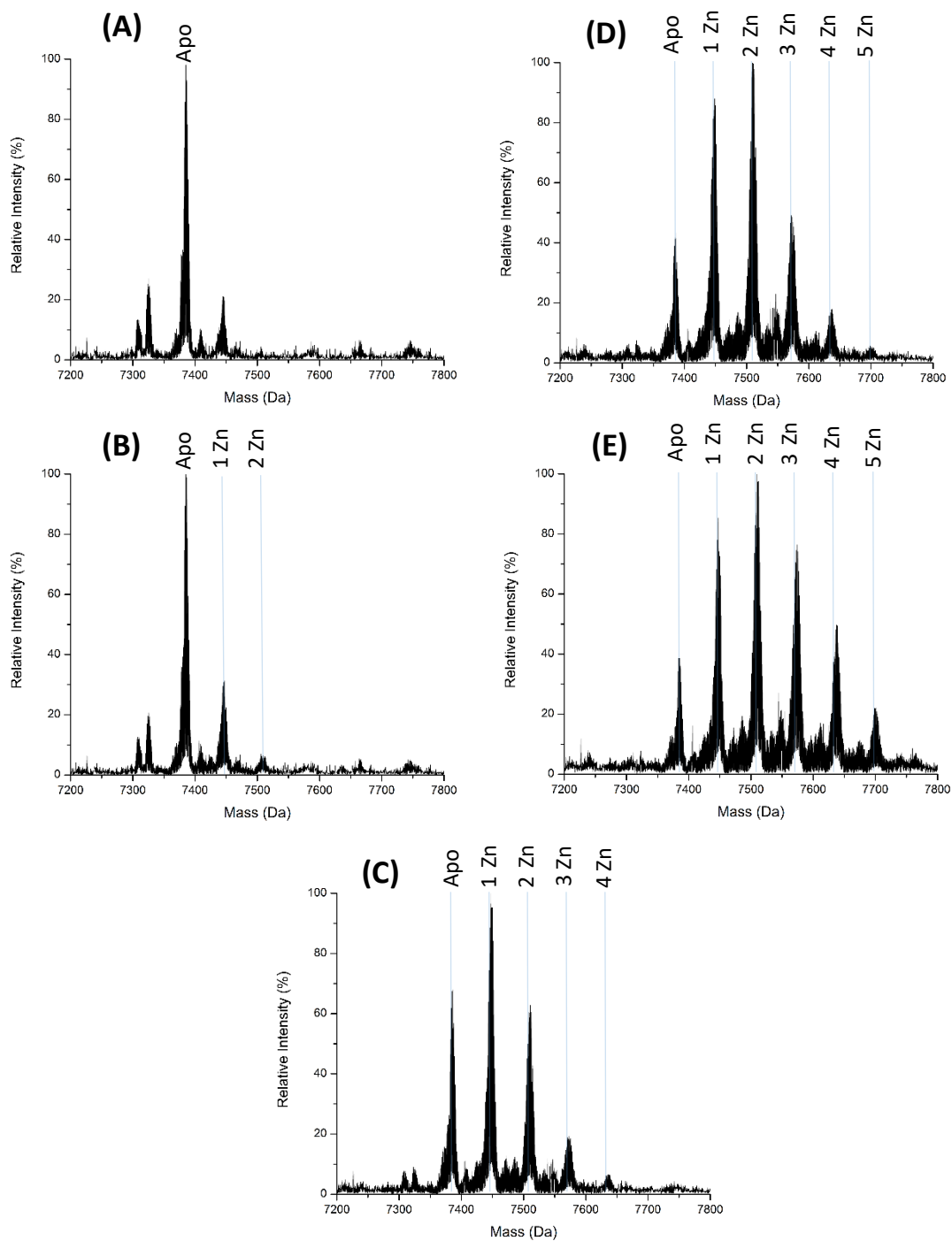


Figure 12. Representative ESI mass spectra for apo-MT2a titrated with zinc acetate between pH 6 and 7. A 0.1 μmol , 0.15 μmol , 0.05 μmol , and 0.075 μmol of 50 mM zinc acetate were added to 312 μM apo-MT2a (B), (C), (D), and (E) respectively.

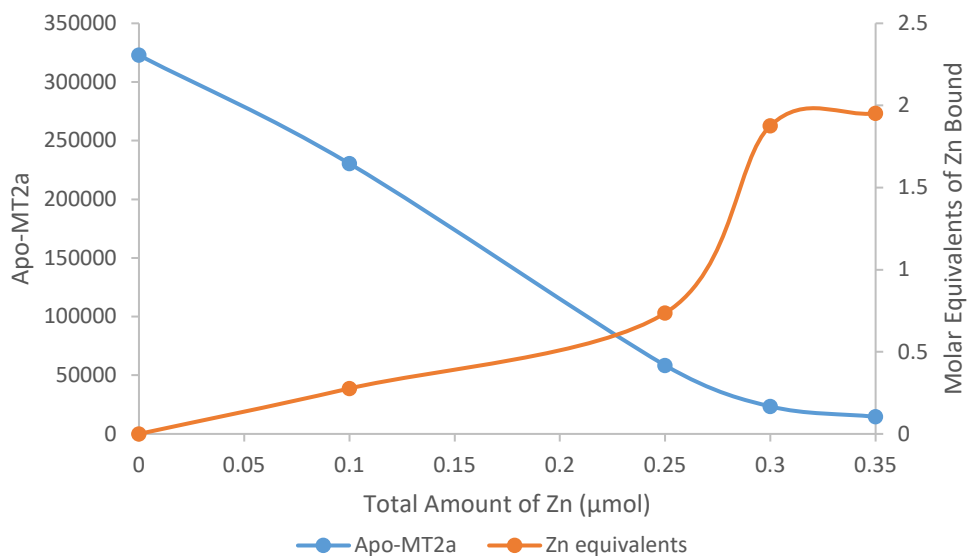


Figure 13. The titration of apo-MT2a with zinc acetate. Apo-MT2a is shown in blue dots and Zn-MT2a is shown in brown dots. Intensity of ESI mass spectral data shown in Figure 12 were used to calculate the molar equivalents of Zn(II). Order of the sequential addition of 50 mM zinc acetate is 0 μmol , 0.1 μmol , 0.15 μmol , 0.05 μmol , and 0.075 μmol (0.35 μmol total zinc acetate in the solution).

3.3.3 pH dependence of zinc binding to apo-metallothionein 2a starting at high pH

A 2.5 molar equivalents of Zn(II) were added to apo-MT2a prior to investigate the pH dependence of Zn binding pathways of MT2a. Terminally bound Zn(II) was observed at pH 7.8 (Fig. 14A), 7.4 (Fig. 14B), 7.1 (Fig. 14C), and 6.1 (Fig. 14D) while a cluster formation dominated at pH 4.7 (Fig. 14G). Mixtures of terminally bound Zn(II) and Zn(II) cluster formation was observed at pH 5.1 (Fig. 14E) and 4.9 (Fig. 14F). At high pH, MT2a showed the non-cooperative metal binding pathway (Fig. 14A-D); while at low pH, MT2a showed a more significant contribution by the cooperative metal binding pathway (Fig. 14G). To examine the pH dependence of metallation of MT2a, the pH of the same solution was changed.

Fraction of Zn_4 clusters calculated using the mass spectral data of Figure 14 and is shown in Figure 15. (Fraction of Zn_4 clusters were calculated by dividing the total intensity of Zn_4 clusters by the total intensity of Zn-MT2a). The cluster formation dominated between pH 4.7 and 5.5 (Fig. 15).

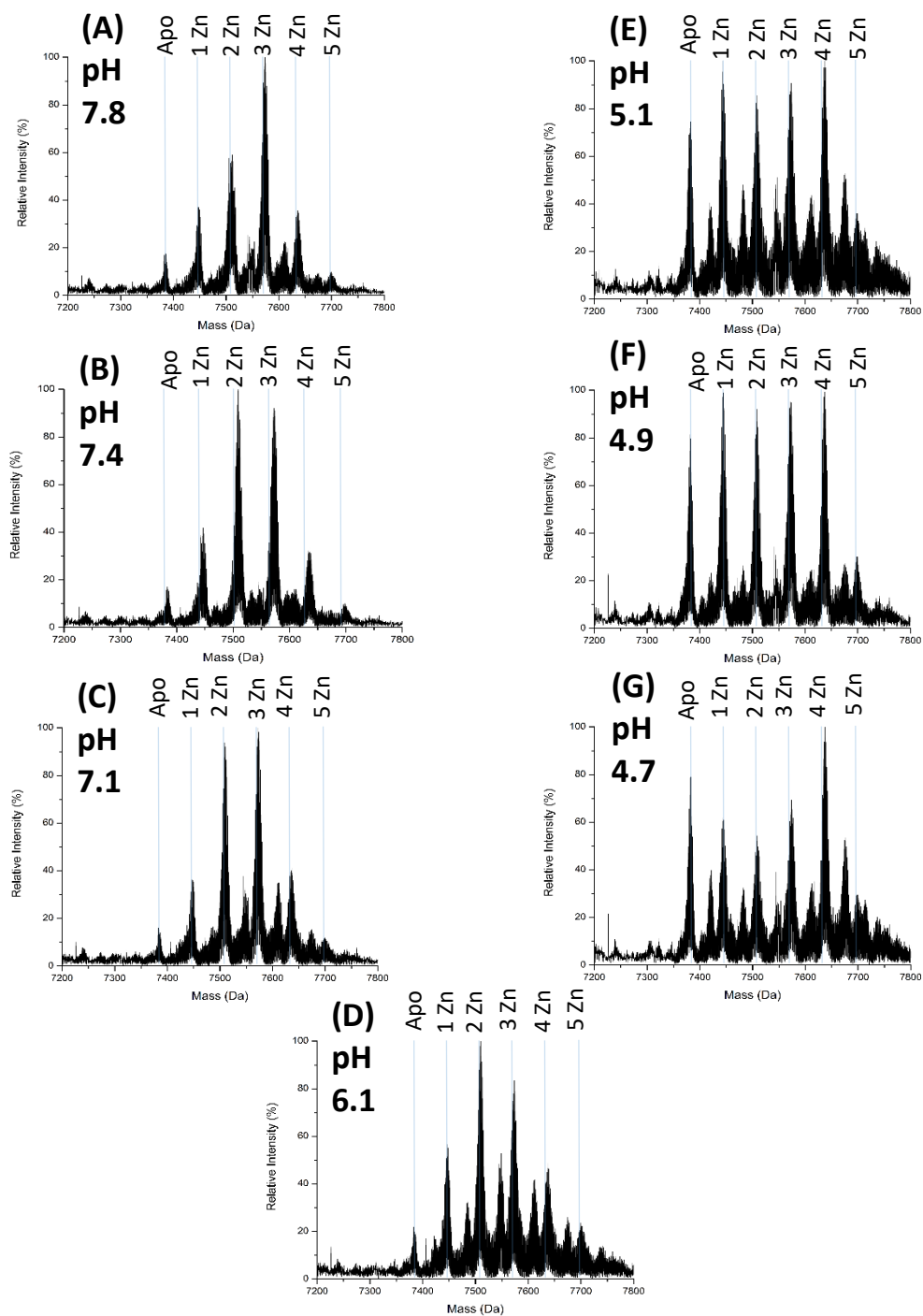


Figure 14. Representative of ESI mass spectra for pH titration of Zn-MT2a. The ESI-mass spectral data were taken during the pH titration from high to low pH after adding the 2.5 molar equivalents of Zn(II).

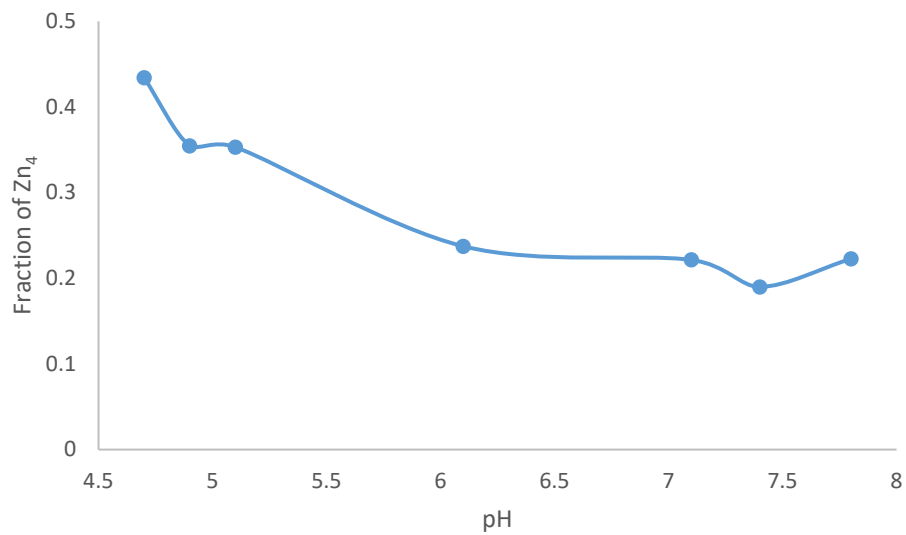


Figure 15. Fraction of Zn₄ cluster formation as a function of pH. The cluster formation pathway was calculated from ESI mass spectral data shown in Figure 14.

3.3.4 Zinc donor-accepter role of metallothionein 2a

Holo-metallothionein 2a transfers metals to apo-MT as well as apo-proteins. To investigate this phenomenon, apo-MT2a was added to Zn-MT2a at physiological pH (7.4) (Fig. 16B). The amount of Zn₇-MT2a, Zn₆-MT2a and Zn₅-MT2a decreased and the amount of Zn₁-MT2a, Zn₂-MT2a and Zn₃-MT2a increased after addition of 300 μ L of apo-MT2a (Fig. 16B). Zinc transfer from Zn-MT2a to apo-MT2a was shown. The persistence of Zn₄ clusters throughout the exchange shows its stability.

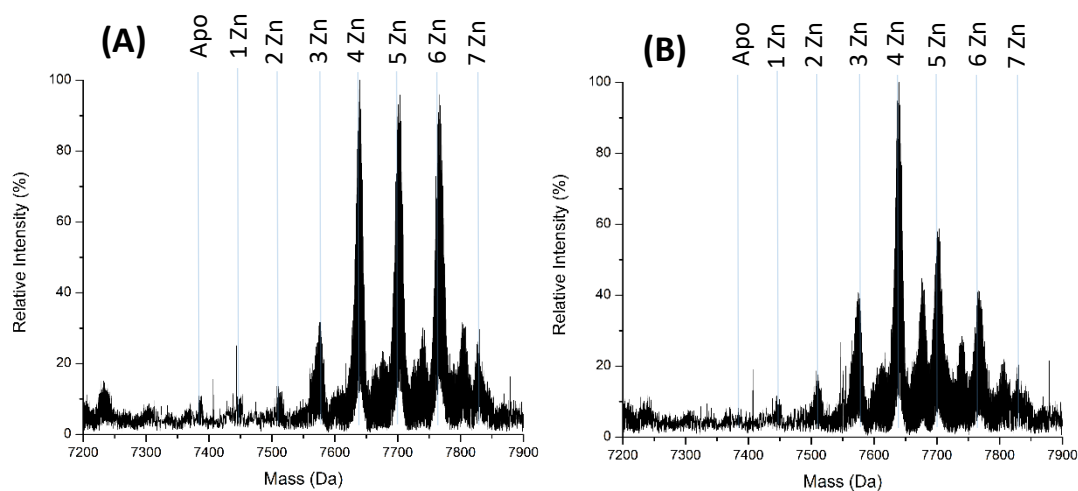


Figure 16. Representative ESI mass spectra for Zn-MT2a at physiological pH 7.4. (A) Zn-MT2a with no apo-MT2a. (B) The spectra of Zn-MT2a, after addition of 300 μ L of 134 μ M apo-MT2a.

Chapter 4

Discussion

4.1 Difficulties in overexpression of recombinant human metallothionein 2a in *E. coli*

Making recombinant protein is usually attractive due to its typical low cost, high purity and high yield. The human *MT2a* gene was inserted into the pET29a plasmid and transformed into *E. coli*. The yield from overexpression of recombinant human MT2a protein was low and could not be optimized using common expression techniques. Because MT2a is made with an S-tag on the N- terminus of the recombinant human MT2a, the N-terminus is not exposed to the outside of the protein. However, the C-terminus is exposed. Metallothionein does not have a β -pleated sheet or α -helix secondary structure like other proteins because its protein folding is metal dependent (Fig. 4). Cadmium was added to the medium to aid in the protein folding, expecting the cysteines to coordinate the Cd(II) in a tetrahedral coordination. Free cysteines are very reactive, and when exposed, they can become modified after demetallation. The primary structure of MT (without Cd) is very prone to oxidation. Because of these reasons, MT2a yield could not be maximized using the sequence available in the Stillman lab. Below, the S-tag is highlighted in blue and the cysteines to be exposed in the C-terminus are highlighted in red:

MKETAAAKFERQHMDSPDLGTLVPRGSMDPNCSCAAGDSCTCAGSCKCKECKC
 TSCKKSCCSCCPVGCAKCAQGCICKGASDK**CSCCA**

4.1.1 Modifying human metallothionein 2a sequence to overcome the overexpression difficulties in *E. coli*

Amino acid residues (KKAAAA) were added to the C-terminus to protect the potentially exposed cysteines. The S-tag was attached to the N-terminus, when MT2a was made in *E. coli*, protecting the N-terminal cysteines. However, the S-tag was cleaved off after purifying the protein, which exposes the N-terminal cysteines. The MT protein is prone to oxidation when Cd(II) is removed from the N-terminus. Therefore, another set of amino acid residues (MGKAAAA) was added to the N-terminus. Below, the S-tag is highlighted in blue, amino acid residues in the N and C termini are highlighted in red and green, respectively.

MKETAAAKFERQHMDSPDLGTLVPRGSMGKAAAAMPNCSCAAGDSCTCAGS
CKCKECKCTSCKKSCCSCPVGCAKCAQGCICKGASDKCSCCAKKAAAA

The higher alanine percent was used when modifying the MT2a sequence because alanine is non-polar and does not carry a charge. The modifications helped to protect the cysteines and the MT2a protein was overexpressed in large quantities in *E. coli*. The mass of the MT2a protein used for the experiments was 59 Da higher than the calculated mass of the protein due to post-translational modification of the protein (Fig. 7 & 8). Because the mass difference of 59 Da is the same as the mass of a carboxymethyl group (59 Da), it is possible that one of the amino acids in MT2a become post-translationally modified by carboxymethylation.

4.1.2 Carboxymethylation of cysteines

Cysteine is one of the most reactive amino acids and is prone to post-translational modifications.⁸⁶ Proteins become oxidized when two cysteines form disulfide bonds. In

addition, amino acids such as cysteine, lysine, methionine, tyrosine and histidine can be modified by carboxymethylation under extreme conditions like pH, temperature and solvent composition.^{87, 88} The addition of a carboxymethyl group to an amino acid yields a modified amino acid that is somewhat similar in structure to aspartic acid.⁸⁷ Therefore, the stability of the native protein remains unchanged.⁸⁹ Due to high cysteine content and reactivity, it is thought that one of the cysteines in recombinant MT2a was post-translationally modified by S-carboxymethylation (Fig. 17).

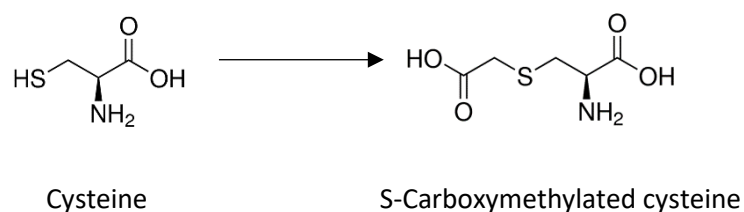


Figure 17. The structures of a cysteine and a carboxymethylated cysteine. The thiol group of a cysteine can be modified by addition of a carboxyethyl group.

4.2 Quantifying the free cysteines in recombinant human metallothionein 2a

Cysteines are involved in the metal binding by MT2a. It is therefore important to quantify the free cysteines, which are cysteines with no disulfide bonds or no post-translationally modified cysteines, in the protein. Quinones react with cysteines in many biological systems.⁹⁰ So, Bq was used to quantify the free cysteines in MT2a (Fig. 18) and it was found that 19 out of 20 cysteines bound with Bq at pH 4.5 (Fig. 9). MT2a with bound *p*-Bq showed a distribution of benzoquinolated species at pH 4.5 (Fig. 9D). It was revealed that one cysteine in MT2a was post-translationally modified by S-carboxymethylation. To break any possible disulfide bonds in MT2a, that may hinder Bq binding, the reducing

agent TCEP was used but the result remained unchanged (Fig. 9F). It was further revealed that MT2a was not oxidized, which means that there were no double bonds between cysteines and one cysteine was post-translationally modified by the addition of a carboxymethyl group.

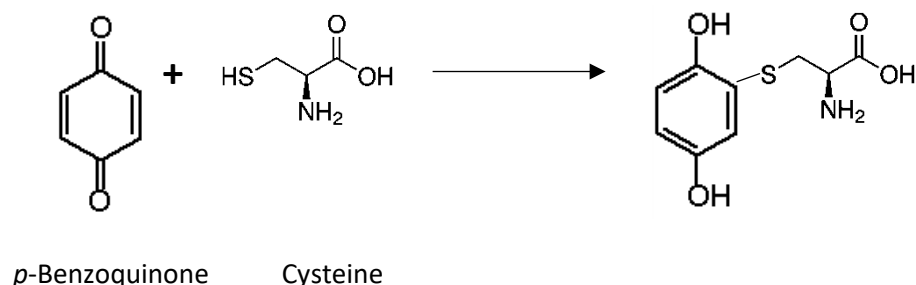


Figure 18. Reaction between a *p*-benzoquinone and a cysteine.

4.3 Zinc binding to apo-metallothionein 2a at low pH

Very acidic environments (pH less than 3) result in demetallation of MT, due to proton competition of the thiols, resulting in apo-MT. At pH 4.1, MT2a did not bind to Zn(II), presumably due to the high proton content in the solution. The metallation of Zn started at pH 4.6 (Fig. 10C) and cluster formation was dominant under these conditions. Even though the metallation of Zn started around pH 4.6 and 4.7, not all Zn ions in the solution were incorporated into apo-MT2, again, presumably due to the high proton concentration. This environment caused the MT2 to exhibit cooperative binding (Fig. 11A). Moreover, MT2a contained more protonated cysteines at low pH. Shifting pH from 4.7 to 5.2 increased the Zn₅-MT2a, Zn₆-MT2a and Zn₇-MT2 species abundance, while lowering the amount of apo-MT2a, Zn₁-MT2a, Zn₂-MT2a and Zn₃-MT2a (Fig. 11B). When the pH of the solution was increased, the concentration of hydroxide ions increased,

which reduced the acidity of the solution. This might be the reason for high Zn binding to MT2a at higher pH.

4.3.1 Zinc binding to apo-metallothionein 2a between pH 6 and 7

Stepwise addition of Zn to apo-MT2a between pH 6 and 7 showed evidence of bead-like structures with Zn binding to terminal thiols, which is indicative of the non-cooperative metal binding pathway (Fig. 12). Several metalloforms with a normal distribution around Zn_1 -MT2a were observed when the MT2a solution contained low Zn concentrations. Then, the Zn distribution shifted over to Zn_2 -MT2a. Sequential addition of approximately 2.0 molar equivalents of Zn(II) caused the amount of Zn-MT2a to increase, while the amount of apo-MT2a decreased (Fig. 13). Apo-MT2a readily picked up the Zn(II) between pH 6 and 7, when Zn(II) was available in the solution.

4.3.2 pH dependence of Zn binding to apo-metallothionein 2a

Zinc binding to apo-MT2a at pH 7.8 also showed evidence for the formation of bead-like structures, which indicates non-cooperative metal binding. Under these conditions, the Zn speciation distribution was skewed with a maximum at Zn_3 -MT2 at pH 7.8 (Fig. 14A). At pH 7.4 and 7.1, Zn speciation distributed normally around Zn_3 -MT2 and Zn_2 -MT2a (Fig. 14B & C). Changing the pH from 7.1 to 6.1 showed that the dominant Zn-MT2a species shifted to Zn_2 -MT2, whereas the non-cooperative metal binding pathway remained unchanged (Fig. 14A-D). Cluster formation (Zn_4 -MT2a) was not dominant in this metal binding pathway, showing less than 50% of the total cluster formation (Fig. 15). At pH 5.2 and 4.9, Zn binding showed the terminally bound Zn(II) as well as cluster formation, indicating a mixture of both binding pathways (Fig. 14E & F). This may be due to the increase in protons in the solution as well as high protonation of cysteines. When the

pH was changed to 4.7, cluster formation (Zn_4 -MT2a) dominated (Fig. 14G). Zinc clusters are very stable because they are formed through the bridging of terminal thiols. The high proton content in the solution may increase the binding affinity of the Zn(II) and cause the formation of very stable clusters in a cooperative manner. This is similar to when oxygen binds to hemoglobin in a cooperative manner, and the binding affinity is regulated by protons, organic phosphates, chlorides and carbon dioxide.⁸⁵ However, *in vitro*, Zn-MT2a cluster formation is regulated by high proton content in the solution.

Irvine *et al.*⁷⁷ showed that Cd(II) binding to MT1a at pH 6.0 formed clusters. Conversely, in my current research, the binding of Zn(II) to MT2a showed that the formation of bead-like structures is dominant over clusters at pH 6.1. I postulate that this is because Cd(II) is toxic to the cell, and is sequestered more readily by MTs to form very stable clusters. However, Zn(II) is an essential trace metal and is required for many Zn proteins. ESI-MS showed that Zn clusters were formed less frequently at pH 6.1 and is mostly distributive, as its ability is important for Zn(II) donation to metalloproteins.

4.3.3 Zinc donor-accepter role of human metallothionein 2a

Even though Zn binding studies were conducted at both high and low pH, it is important to look closely at the Zn binding pattern at the physiologically relevant pH of 7.4. Several Zn-MT2a metalloforms exist at pH 7.4 (Fig. 16A). One of the most important factors of MT is its Zn donor-accepter role in biological systems. Metallothionein donates Zn(II) to other Zn proteins and it picks up excess Zn(II) from the cytosol, as part of the tight control of free Zn levels in cells. After addition of apo-MT2a to the holo-MT2a at pH 7.4, the amounts of Zn_5 -MT2a, Zn_6 -MT2a and Zn_7 -MT2a decreased while the amounts of Zn_1 -MT2a, Zn_2 -MT2a and Zn_3 -MT2a increased indicating the transfer of Zn(II) from holo-

MT2a to apo-MT2a (Fig. 16). The clusters of four Zn bound to MT2a were very stable and the relative amount of Zn₄-MT2a remained unchanged (Fig. 16).

4.4 Importance of this research

Only a few published papers have discussed the expression of human MT2a in *E. coli*⁹¹⁻⁹³ but they did not conduct any metal binding studies using that protein. Also, not very many Zn binding studies have been conducted using human MT2a. My research has made three important discoveries:

1. At low pH (4.7), MT2a formed very stable Zn clusters.
2. At physiological pH (7.4), MT2a formed bead-like structures.
3. At physiological pH (7.4), holo-MT2a donated Zn to apo-MT2a, which confirms the donor-acceptor role of MT2a.

My research is very important because it leads towards a better understanding of liver MT2a and how it is similar to kidney MT1a. Also, my findings verified the well-known phenomenon of isoforms, where the isoforms are similar proteins and perform the same function.

4.5 Future work

The next step is to look at Cd, As and Cu binding pathways of MT2a. Then, we can compare the metal binding mechanism of MT2a with the metal binding mechanism of MT1a, which was examined by the Stillman group over the last few years.^{47,48,77,82} From this we can determine if the metal binding of MT2a is different or similar to MT1a, even though they are two isoforms.

4.6 Summary and conclusion

The goal of this study was to overexpress recombinant human MT2a in *E. coli* in large quantities, characterize it, and determine its Zn binding pathway. The Bq studies showed that one out of 20 cysteines in MT2a was post-translationally modified, while the 59 Da mass shift of both the Cd₇-MT2a and apo-MT2a suggested a single carboxymethylation. In addition, donation of Zn(II) from holo-MT2a to apo-MT2a at physiological pH (7.4) confirmed the donor-accepter role of MT to other proteins in nature.

Zinc binding studies of MT2a showed that at physiological and higher pH, MT2a followed a non-cooperative metal binding pathway, which means that Zn binds first with terminal thiols, making bead-like structures with no significant impact of one atom of Zn binding affecting the affinity of the protein for other Zn atoms, before adopting a final folded conformation. At low pH, MT2a showed cooperative metal binding, which means that stable Zn clusters were formed through the bridging of terminal thiols. However, at low pH, Zn binding to MT2a did not show fully cooperative (100%) manner because intermediates of Zn₁-MT2a, Zn₂-MT2a, Zn₃-MT2a and Zn₅-MT2a were also seen in the solution even though Zn clusters were dominant.

While previous work in the Stillman lab was carried out using kidney MT1a,^{47, 50, 52} it was important to understand the role of the recombinant human MT2a from the liver. Metallation properties of the two proteins are expected to be different due to the differences in the two amino acid sequences. However, Zn binding to MT2a described in this thesis was similar to Zn binding to MT1a that has been reported in literature,⁷⁷ showing that the two isoforms behave similarly even though their amino acid sequences are slightly different.

I hypothesized that metallation will differ between MT2a and MT1a due to their sequence differences. In my current research, the Zn binding data of MT2a showed that at low pH, Zn(II) binds cooperatively to MT2a forming stable Zn clusters and at physiological pH, Zn(II) binds non-cooperatively to MT2a forming bead-like structures. Irvine *et al.*⁷⁷ reported that at low pH, Zn(II) binds cooperatively to MT1a and at physiological pH, Zn(II) binds non-cooperatively to MT1a. Therefore, I conclude that the Zn binding of MT2a is similar to the Zn binding of MT1a. Forming bead-like structures at physiological pH, both MT2a and MT1a can donate Zn(II) to other Zn-containing proteins and this is important to conduct biological functions more efficiently.

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