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The role of metallothionein in zinc homeostasis

Kelly L. Summers
The University of Western Ontario

Supervisor
Mark Bernards
The University of Western Ontario

Graduate Program in Biology

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

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THE ROLE OF METALLOTHIONEIN IN ZINC HOMEOSTASIS

(Thesis format: Monograph)

by

Kelly Lynn Summers

Graduate Program in Biology

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The structure of the unique metal-binding protein, metallothionein (MT), consists of two metal-thiolate-clustered binding domains; the β-domain binds up to three divalent metals and the α-domain binds four. The mechanisms through which the metals are bound and arranged into domains, as well as the function of MT in metal ion homeostasis, remains largely unknown. By utilizing electrospray ionization mass spectrometry (ESI MS) to identify each species, and by comparing the data with simulations, MT 1a was found to bind Zn$^{2+}$ non-cooperatively. Through a competition experiment between MT and its individual domain peptides, MT was proposed to bind Zn$^{2+}$ using terminal cysteines initially (into five strong binding sites) before formation of the clusters with binding of the sixth and seventh Zn$^{2+}$ (into two weaker binding sites). The terminally bound Zn$^5$-MT intermediate is thought to be key to metal acquisition (from zinc-chaperones) and donation (to zinc-dependent apo-enzymes) in maintaining intracellular zinc homeostasis.

Keywords: metalloproteins, zinc metallothionein, zinc binding proteins, cellular zinc buffering, zinc homeostasis, metallation intermediates, non-cooperative metallation, metal ion competition, electrospray ionization mass spectrometry, metal ion transfer, protein structure, protein function
Co-Authorship Statement

This thesis contains material from the following published manuscripts:


Martin J. Stillman is the corresponding author on all presented papers and was responsible for supervising me during my studies. For all the results presented in this thesis, I carried out all of the experimental work, prepared the experimental data for publication, and completed all the modelling calculations. More specifically, I began the experimental work with the expression of the recombinant proteins (Subsection 2.1.2), continued through the purification steps (Subsection 2.1.3 – 2.1.6) to the characterization of Zn$^{2+}$ binding using electrospray ionization mass spectrometry and molecular modelling (Sections 2.2 and 2.3, respectively). Earlier experimental work, mainly the results presented in Section 3.1, was conducted under the guidance of senior graduate student, Duncan E. K. Sutherland. The questions to be investigated were initially conceived by Martin in consultation with Duncan (Section 3.1), and later, in consultation with me (Section 3.2). After I had plotted and presented the data, discussions between Duncan, Martin, and I resulted in the interpretations of the data. In all three publications, I prepared the figures and assembled them into a logical order. Duncan generated the spreadsheet that was then used (by Duncan in the second paper and by me in the third paper) to model the binding constants of each Zn$^{2+}$ binding site. Duncan, Martin, and I all had considerable input into the writing of the manuscripts for publication. Although Duncan was largely responsible for preparing the initial drafts, Martin and I edited the papers heavily before publication. Furthermore, the third paper was almost entirely re-written and re-organized by me before submission.
Acknowledgments

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I would like to thank Dr. Duncan Sutherland for training me on all the quirks of protein purification and how not to oxidize my protein before I even started the experiments. Thanks must also be given to the ‘boys’ in the Stillman group – Gordon Irvine, Tyler Pinter, Dr. Duncan Sutherland, and Dr. Michael Tiedemann – for assuming there must be something wrong with my data or interpretation and thus forcing me to go to great lengths to prove my point and ultimately strengthening my conclusions. Finally, I must give special thanks to my closest friends – Tasha Jarisz, Kyle Jeffs, Jocelyn Kennedy, and Melanie Vaillancourt – and family for proofreading my arduous rough draft, and for being there (even from the other side of the country) and believing in me when I needed you the most.
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<th>Definition</th>
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<tbody>
<tr>
<td>α-rhMT 1a</td>
<td>Recombinant alpha domain peptide of human metallothionein isoform 1a</td>
</tr>
<tr>
<td>β-rhMT 1a</td>
<td>Recombinant beta domain peptide of human metallothionein isoform 1a</td>
</tr>
<tr>
<td>βα-rhMT 1a</td>
<td>Recombinant, two domain, human metallothionein isoform 1a</td>
</tr>
<tr>
<td>apo-protein</td>
<td>Protein without its respective metal ion cofactor</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism spectroscopy: Measures differences in absorption of left-handed and right-handed polarized light arising from structural asymmetry</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory: A quantum mechanical modelling method used to investigate electronic structures of atoms and molecules</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance: A spectroscopic technique used to study molecules with unpaired electrons</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization: A soft ionization method used in mass spectrometry to generate ions</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray absorption fine structure: A technique that provides information on the local structure surrounding a specific element of interest</td>
</tr>
<tr>
<td>holo-protein</td>
<td>Protein bound to its respective metal ion cofactor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside: A reagent used to induce expression of genes controlled by a lac operator</td>
</tr>
<tr>
<td>K_F</td>
<td>Formation constant: The equilibrium constant for the formation of a complex from its components in solution</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth: A bacterial growth medium</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography: A technique used to separate molecules using a liquid mobile phase</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry: An analytical technique that provides the masses of the complexes in a solution</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein: A small, cysteine-rich, metal-binding protein</td>
</tr>
<tr>
<td>MTF1</td>
<td>Metal response element-binding transcription factor 1: A zinc finger protein that induces transcription of metalloproteins</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance: A spectroscopic technique used to determine the physical and chemical properties of atoms or molecules by exploiting the magnetic properties of certain atomic nuclei (e.g., $^{113}$Cd and $^1$H)</td>
</tr>
<tr>
<td><strong>OD</strong></td>
<td>Optical density: A measure of the amount of light absorbed by a solution compared to the amount of light transmitted through a solution</td>
</tr>
<tr>
<td><strong>Tris</strong></td>
<td>Tris(hydroxymethyl)aminomethane: Used as a buffer with a pKa of 8.0 at 25°C (i.e., it is effective over a pH range of 7.1 - 9.0)</td>
</tr>
<tr>
<td><strong>rhMT 1a</strong></td>
<td>Recombinant human metallothionein isoform 1a</td>
</tr>
<tr>
<td><strong>UV</strong></td>
<td>Ultraviolet light: Electromagnetic radiation with shorter wavelengths than visible light (&lt; 400 nm) and longer wavelengths than x-ray light (&gt; 10 nm)</td>
</tr>
<tr>
<td><strong>ZAP1</strong></td>
<td>A zinc-responsive influx regulator that binds to the ZRE sequence upstream of zinc-regulated genes (such as ZIPs and ZRTs)</td>
</tr>
<tr>
<td><strong>ZiaR</strong></td>
<td>A zinc-responsive repressor of heavy metal efflux transporter, ZiaA</td>
</tr>
<tr>
<td><strong>ZIPs</strong></td>
<td>Zinc influx transporters (also known as ZRT, IRT-like proteins, and SLC39)</td>
</tr>
<tr>
<td><strong>ZnTs</strong></td>
<td>Zinc efflux transporters (also known as CDF or SLC30)</td>
</tr>
<tr>
<td><strong>ZnTR</strong></td>
<td>A zinc-responsive repressor of a zinc efflux pump, ZnTA</td>
</tr>
<tr>
<td><strong>ZUR</strong></td>
<td>A zinc-responsive uptake regulator that represses transcription of zinc uptake systems</td>
</tr>
<tr>
<td><strong>ZRE</strong></td>
<td>The zinc responsive element sequence, which is bound by zinc sensors</td>
</tr>
</tbody>
</table>
1 Introduction

1.2 Metals in Biology

Biological systems are composed of elements readily found in the environment. Aside from the obvious elements that make up the majority of organic structures and metabolites (i.e., carbon, hydrogen, oxygen, and nitrogen), additional elements are essential in large amounts (e.g., sodium, potassium, calcium, magnesium, phosphorus, sulfur, iodine, and chlorine) or in smaller amounts (e.g., manganese, iron, cobalt, nickel, copper, zinc, molybdenum, boron, silicon, and selenium) by most, if not all, biological systems (1). Other elements are potentially essential for a select few organisms (e.g., barium, tungsten, cadmium, tin, arsenic, and bromine) (1).

Throughout evolution, the variety and abundance of metals available to organisms has changed. In particular, the liberation of dioxygen through photosynthesis had an enormous impact on the solubility of metals (1). Prior to the shift toward an oxygenic atmosphere, the concentration of sulfide in the ancient oceans was high and, therefore, the majority of metal ions were in metal sulfide complexes (2). Under these conditions, copper, zinc, and cadmium were inadequately available to organisms, while cobalt, nickel and iron were more available for use in enzymes (2). As levels of oxygen in the atmosphere increased, copper and zinc became more soluble, but cobalt and iron formed hydroxyl complexes and thus became less bioavailable (2). Metal availability in the archaic oceans is an important consideration when contemplating the trace metal composition of organisms because the biological chemistry inside cells is essentially chemistry in water.

Changes in the bioavailability of metals can be inferred from the utilization of metals in increasingly more complex organisms as they evolved. Bacteria and archaea evolved in the ancient oceans under anoxic conditions where iron was soluble (i.e., ferrous iron, Fe$^{2+}$) and therefore these organisms exploited the redox potential of iron in many enzymes. Bacteria and archaea have proportionally more iron-sulfur proteins, but fewer heme proteins than eukaryotes (3). In the oxygenic environment present today, iron is
poorly soluble (i.e., ferric iron, Fe$^{3+}$) and, therefore, plants and bacteria use siderophores and reductases to scavenge what little iron is available (4, 5).

As eukaryotic genomes became increasingly more complex, zinc and copper were used in an increasing number of metalloproteins (2, 6, 7). Zinc is probably most commonly known for its role in zinc finger proteins; zinc finger motifs are prevalent in transcription factors, which control the expression of genes associated with differentiation of cell types (2). Copper proteins are most commonly used in electron transport chains for aerobic respiration and photosynthesis (7). In addition, concentrations of both zinc and copper are maintained in the cytoplasm of cells through metal importers, exporters, and metal storage proteins (2).

1.2.1 Essential Metals

Thirteen metals are known to be required in specific quantities for the proper functioning of plants and animals (8). Group one and two metals (i.e., sodium, potassium, magnesium, and calcium) are required in large amounts and are thus referred to as bulk metals. The d-block metals (i.e., vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum) are required in smaller quantities and are thus referred to as trace metals (1). Much is known about the bulk metals and their roles in mammalian systems possibly through the sheer quantities of these elements in animals, but much remains unknown about some of the trace metals and their roles in metabolism.

Manganese is well known for its role in the oxygen-evolving complex of photosystem II, but also has a number of other important roles including those in glycosylation and as an antioxidant (1, 9, 10). Iron is well known for its role in hemoglobin and the transport of oxygen in animals, although iron is a cofactor in both heme and non-heme proteins, which catalyze a great number of reactions in plants and animals (such as nitric oxide and carbon dioxide transport, as well as electron transport) (11, 12).

Copper and zinc increased in bioavailability after atmospheric dioxygen appeared in substantial quantities. Because of its availability and because of its redox potential, copper is found in all plants and animals as an essential electron donor and acceptor in a
multitude of cuproproteins; copper is key to energy production in most eukaryotes (1). Because free copper can be highly detrimental, a number of proteins and enzymes are responsible for regulating intracellular copper concentrations (1). Zinc is essential to almost all species. Arguably, the most important roles of zinc are in development, growth, and healing, with particularly significant functions in DNA replication and translation, and mammalian respiration (1, 13). Similar to copper, the abundance of zinc is tightly regulated in living systems; however, this is more likely due to the wide usage of zinc and the severe effects of zinc deficiency rather than any detrimental consequences of free zinc.

1.2.2 Toxic Metals

A number of metals are known to be toxic to humans and other mammals (e.g., lead, arsenic, mercury, cadmium, and uranium), although all the essential metals can become toxic at high concentrations. Some metals are essential at one oxidation state and toxic at another (e.g., chromium (III) is essential; chromium (VI) is toxic (14)) or some metals may be detrimental, or more detrimental, in complexes (e.g., mercury is generally toxic, but methyl mercury is one of the most toxic metallocomplexes (15)). Some toxic metals, such as lead, mercury, and cadmium are thought to enter cells through essential metal transporters or pumps (16). Some metal ions may also pass through the cell membrane alone or in complex with proteins or small molecules (16). Once inside the cell, toxic metals often outcompete essential metals for binding sites in enzymes (e.g., cadmium can easily replace zinc (15, 17)), causing the toxic effects.

Lead can replace calcium, leading to the deposition of lead in bone, and learning deficiencies when lead replaces calcium in protein kinase C (an important protein in signal transduction pathways) (18). Lead can also replace zinc and iron in enzymes leading to developmental delays, learning disabilities, and behavioural problems in children (18, 19). Possibly the most adverse effect of lead exposure is nervous system damage; lead can cross the blood-brain barrier and lead to encephalopathy (19).

Depending on the mode of exposure and whether exposure is acute or chronic, arsenic can cause a number of health problems (e.g., skin lesions, pulmonary edema, and liver
failure) and is a known carcinogen (20). Arsenic uptake occurs through phosphate, glucose, and glycerol transport pathways in prokaryotes and eukaryotes (21). Arsenic causes damage through multiple mechanisms, including oxidative stress, cell membrane disruption, and destruction of hemoglobin (20). Surprisingly, some evidence suggests that arsenic may be essential to some organisms (22).

Cadmium is toxic to plants, causing symptoms such as growth retardation, inhibition of photosynthesis, and generation of free radicals to name a few (23). Some plants have adapted to cadmium-contaminated environments by accumulating or sequestering cadmium, synthesizing phytochelatins (i.e., metal ion chelators) and inducing heat shock proteins (23). In herbivores, the effects of cadmium toxicity can been seen in the lungs, heart, liver, and kidneys (15). The majority of the toxic effects of cadmium exposure may be attributed to the substitution of cadmium in zinc enzymes, although cadmium also interferes with redox sensitive systems (15, 17). The latter is attributed to the replacement of iron in iron-requiring redox enzymes (15, 17).

1.3 Homeostasis of Metals

The concentrations of metal ions, like many other compounds, are usually maintained within a set range regardless of the concentration of metals in the environment. Proteins that bind metals (metalloproteins) are required to maintain this equilibrium. If intracellular metal ion concentrations are not maintained at precise levels, a number of diseases and detrimental effects may result. For example, in mammals, copper imbalances may lead to Wilson’s or Menkes disease (24), zinc deficiencies may lead to growth retardation (25), and iron imbalances may lead to haemochromatosis or anemia (26).

1.3.1 Role of Metalloproteins

More than 30% of all proteins utilize a metal ion to perform their function and almost 50% of all enzymes contain a metal cofactor (2, 8). Metal ion cofactors are often crucial to protein structure and function. These cofactors may aid in the catalysis of reactions, act as electron acceptors or donors, or serve an exclusively structural function. Other proteins bind essential metals to transport, store, or otherwise regulate the concentration
of metal ions available to a cell. Proteins bound to their respective metal ion are referred to as ‘holo’; proteins without their cofactor are referred to as ‘apo’.

Iron and copper are redox-active metals (i.e., can switch between oxidized and reduced forms) and are often involved in electron transfer (e.g., heme-binding cytochromes and blue-copper proteins in respiration and photosynthesis) and in the storage and transport of dioxygen (e.g., hemoglobin, myoglobin, and hemocyanin) (2, 27). Zinc is a superacid center in metalloproteins that promotes hydrolysis or cleavage of chemical bonds. Enzymes such as carboxypeptidases, carbonic anhydrase, and alcohol dehydrogenase contain catalytic zinc sites; however, many proteins contain zinc sites that have an exclusively structural role (e.g., superoxide dismutase and zinc fingers) (2). Other trace metals have also been identified as cofactors in metalloproteins. For example, manganese is a cofactor in mitochondrial superoxide dismutase, inorganic phosphatase, and photosystem II; nickel functions in enzymes such as urease and numerous hydrogenases; and molybdenum and vanadium are found in nitrogenases, along with iron (2).

Proteins that store and transport metals, are required to maintain metal ion homeostasis. For example, in mammals, copper is believed to be absorbed from the stomach and small intestine by metallothionein and albumin, transported through the blood via albumin, and delivered to the organs that require copper (i.e., mainly the liver, brain, heart, and kidneys) (24). At the cellular level, copper is delivered to membrane-bound transporters (e.g., eukaryotic CTR or bacterial CopA) by copper chaperones (e.g., bacterial CopZ) (28-30). Once inside the cell, copper is transferred via a number of chaperones before being inserted into the Cu-dependent apo-enzyme. In yeast, Cox17 delivers copper to the intermembrane space of the mitochondria to be incorporated into cytochrome c oxidase (i.e., the last enzyme in the electron transport chain of aerobic respiration), and CCS delivers copper to cytoplasmic superoxide dismutase (detoxifies superoxide radicals utilizing the redox cycling of bound copper) (30).
1.3.2 Structure of Metal Binding Sites

Amino acids that regularly act as metal ligands in proteins are the thiolates of cysteines, the imidazoles of histidines, the carboxylates of glutamic and aspartic acids, and the phenolates of tyrosines (8). Metal cofactors act as Lewis acids and protein ligands act as Lewis bases (31), generally following the hard-soft theory of acids and bases (i.e., the Pearson acid base concept (32, 33)). This concept is often used in transition metal chemistry to characterize the stability of compounds, reaction mechanisms, and pathways. The term 'hard' describes small, highly charged, weakly polarizable species, whereas the term 'soft' applies to large, low-charge species that are strongly polarizable (34). Essentially, the theory predicts that soft acids (e.g., metals such as Cu\(^+ \) and Cd\(^{2+} \)) will react faster and form stronger bonds with soft bases (e.g., S\(^- \) from cysteine), whereas hard acids (e.g., Mg\(^{2+} \) and Ca\(^{2+} \)) will react faster and form stronger bonds with hard bases (e.g., OH\(^- \) from tyrosine, or NH\(^+ \) from histidine).

Proteins may perform different types of biological functions, depending on the metal they contain and its coordination. The coordination number and geometry of the metal site is determined by the oxidation state of the metal, although distortions from the expected structures often occur in metalloproteins. For example, Cu\(^{2+} \) favours tetragonal coordination, whereas Cu\(^+ \) prefers tetrahedral or trigonal planar geometries (35). Many copper chaperones in yeast (like those discussed above) have a GMXCXXC copper-binding motif, in which the two cysteines bind Cu\(^+ \) in a low-coordination-number environment (36). This coordination allows for tight binding while still allowing copper transfer between binding domains of individual copper-binding proteins (36). In blue copper proteins, which catalyze redox reactions in many organisms, another unexpected copper binding site geometry is evident. From a crystal structure of plastocyanin, the copper is believed to be bound by a cysteiny1 thiolate, two histidine residues, and possibly a rather distant methionine sulfur (35). The binding sites of blue copper proteins appear to be constrained such that the geometry does not change upon oxidation or reduction of the copper ion (35). This is thought to aid in the redox activity of the metal center by ensuring that neither conformation is more stable than the other.
Zinc is considered a ‘borderline’ metal and therefore binds a range of soft, hard, and borderline ligands, including the carboxylate groups of aspartate and glutamate, phosphates, histidine imidazoles, cysteine thiolates, and even water or hydroxide ions (37). Depending on the ligands, the zinc site may simply aid in the structure of the protein, or the zinc site may facilitate reaction catalysis. In structural sites, where Zn$^{2+}$ is usually bound to a mixture of nitrogen from histidine and sulfur from cysteine, the coordination number is usually four and Zn$^{2+}$ is usually bound in a tetrahedral geometry (38). In catalytic sites, where Zn$^{2+}$ is bound to water in addition to nitrogen from histidine, sulfur from cysteine and, often, oxygen from either aspartic or glutamic acid, the coordination number may increase from four to five, or from five to six (38).

### 1.3.3 Binding Mechanisms

Exactly how metals are bound or inserted into proteins is a topic currently receiving quite a bit of attention. Some metallochaperones transfer metal ions directly from influx transporters on the cell membrane to metalloproteins that require them to function (e.g., the copper chaperones mentioned above) although others do not appear to require chaperones. For many proteins, the mechanism of metal binding remains unknown, often because of the many problems of studying proteins in vitro in an attempt to elucidate in vivo reactions.

Metalloproteins that bind more than one metal or substrate molecule(s) may bind through different mechanisms that can be measured in kinetics studies. Binding constants ($K_F$s) are used to describe the binding site affinity, or how strongly the substrate is held within a binding site. If binding of the first substrate increases the affinity ($K_F$) for additional molecules at another site, substrate binding exhibits positive cooperativity (39). Conversely, if binding of the first substrate lowers the $K_F$ of subsequent molecules, substrate binding exhibits negative cooperativity (39). If substrate binding at each site is independent of the others, the binding is non-cooperative.

Most enzymes do not show any cooperativity in substrate binding, but there are a number of examples of both positive and negative cooperativity in substrate binding by various proteins (40). Oxygen binding to hemoglobin is one example of positively cooperative
substrate binding \((41)\). Hemoglobin is a tetrameric protein that contains four hemes (i.e., one in each subunit), each of which binds a dioxygen molecule. Binding of the first dioxygen increases binding of additional oxygen molecules, such that the fourth and last dioxygen binds with the highest affinity \((K_F)\).

ATP-citrate lyase is an example of an enzyme that exhibits negative cooperativity in binding its substrate, citrate. ATP-citrate lyase is a tetrameric enzyme with four identical subunits; it is a key enzyme in the reductive tricarboxylic acid cycle, which is the carbon dioxide fixation pathway in bacteria \((42)\). ATP-citrate lyase uses ATP to catalyze the conversion of citrate and CoA into acetyl-CoA and oxaloacetate \((42)\). Citrate binding to ATP-citrate lyase demonstrates negative cooperativity; binding of the first and second citrate molecules significantly decreases the affinity of the enzyme for subsequent citrate molecules \((42)\).

1.4 Zinc Homeostasis

Zinc, the most abundant trace metal ion in aerobic cells, is an essential cofactor of numerous enzymes, which have roles in a wide range of biochemical pathways. For example, \(\text{Zn}^{2+}\) is a structural component in growth factors, cytokines, receptors, and transcription factors belonging to a number of cellular signaling pathways \((43, 44)\). Zinc thus has a role in a large number of cellular processes from DNA and protein synthesis to mitosis and cell division \((45)\). Because \(\text{Zn}^{2+}\) is vital to so many processes, levels are tightly regulated both at the cellular level and at the organismal level. Zinc binding in enzymes is very strong, with binding constants on the order of \(10^{11} \text{M}^{-1}\) \((1)\). Cellular \(\text{Zn}^{2+}\) levels are tightly controlled by transporters \((46)\), channels \((47)\), \(\text{Zn}^{2+}\) sensing storage proteins (i.e., MTs; \((48)\)), and transcription factors (e.g., MTF-1; \((25, 49)\)), such that the concentration of free \(\text{Zn}^{2+}\) is about \(10^{15} \text{M}\) in the cytoplasm (i.e., approximately one unbound \(\text{Zn}^{2+}\) per cell) \((50)\). When levels of \(\text{Zn}^{2+}\) are unregulated, a number of health problems, such as growth retardation, immunodeficiency, and neuronal and sensory dysfunctions result \((25)\). Because zinc is directly involved in tissue synthesis, manifestations of zinc deficiency in humans can be as severe as dwarfism \((51)\).
One major role of zinc in eukaryotes is in gene regulation through proteins containing a zinc finger motif. In this way, the structural role of zinc extends to the organization of chromosomes (52). Most zinc fingers bind to DNA promoters, but the zinc cofactor appears to have a structural or regulatory role. Zinc finger proteins regulate gene expression, and as an extension mRNA and proteins, in a variety of pathways involved in cell differentiation, the cell cycle, and even apoptosis and cancer (53). From the diversity of proteins containing zinc finger motifs, and thus containing structural zinc-binding sites, it is easy to see how zinc deficiencies can cause such profound effects.

The total zinc content of a cell is believed to be approximately 0.1-0.5 mM across a number of study species including Escherichia coli, yeast, and mammalian cells, although essentially all the zinc is bound to proteins (54). The mechanism(s) that controls the cellular zinc concentration has been the focus of much research since the characterization of the first zinc transporter (55). Zinc transporters, responsible for zinc influx and efflux, are thought to be key to maintaining intracellular zinc concentrations. However, influx and efflux are not only important for maintaining the concentration of zinc within a cell, but also for transporting zinc through epithelial cells in the intestine into the bloodstream. Studies have also described zinc-sensing transcription factors that control the influx and efflux of zinc into the cell (2). Hantke (56) provides an excellent summary of the many zinc-binding proteins – including proteins involved in influx and efflux – in the periplasm of Escherichia coli.

1.4.1 Zinc Influx

Because zinc is required in so many intracellular pathways and proteins, cells must have mechanisms dedicated to bringing zinc across the cell membrane. Zinc influx proteins are membrane-bound transporters that facilitate the movement of extracellular Zn$^{2+}$ into the cytoplasm. A number of different study species from bacteria to yeast to mammalian cells have been examined in an attempt to elucidate the mechanisms of zinc influx into cells. Each system has its own nomenclature, although the proteins are highly similar. A number of zinc uptake systems, including the bacterial Znu proteins, the eukaryotic ZIP proteins, and the yeast ZRT proteins, are thought to be essential in maintaining zinc homeostasis.
A high-affinity zinc-uptake system has been found in many bacterial species. Three proteins encoded by the *znuA*, *B* and *C* genes have been identified; these include a periplasmic binding protein (ZnuA), a membrane permease (ZnuB), and an ATPase (ZnuC) (56, 57). Expression of this system is believed to be regulated by zinc as well as a bacterial repressor from the FUR family known as ZUR (57).

ZIPs (also known as ZRT, IRT-like proteins) have been found in many eukaryotes including animals, plants, protists, and fungi. ZIPs were named for their similarity to both the ZRT (the zinc regulated transporter) and IRT (the iron regulated transporter) proteins described prior to the discovery of ZIPs. ZRT1 and ZRT2 are ATP-dependent high- (K_F of approximately $10^8$ M$^{-1}$) and low-affinity (K_F of approximately $10^7$ M$^{-1}$) zinc transporters in *Saccharomyces cerevisiae*, respectively (58, 59). Similarly, IRT1 is an iron transporter in *Arabidopsis thaliana* (60).

Mammalian ZIPs are officially known as the SLC39 family of metal ion transporters and, unlike yeast ZRT proteins, they do not appear to require ATP. The ZIP protein family transports zinc (and/or other metal ions) from the extracellular space or from the lumen of organelles into the cytoplasm (54). This protein family is large with an estimated 14 ZIPs with differing localizations within cells and within organs.

The ZIP1 (SLC39A1) transporter is ubiquitously expressed in mice and human tissues and is thought to be important in zinc uptake in a variety of cell types (61, 62). ZIP2 (SLC39A2) and ZIP3 (SLC39A3) are also believed to have roles in zinc uptake by mammalian cells (63-65). ZIP3 appears to be particularly important in zinc uptake by mammary epithelial cells to provide the milk with zinc (66). ZIP4 (SLC39A4) appears to be an important transporter for zinc absorption from the intestine; mouse ZIP4 has been localized to the apical membrane of the absorptive intestinal cells (54, 67). ZIP5 (SLC39A5) also appears to be important in zinc absorption, although ZIP5 is expressed on the basolateral membrane of intestinal enterocytes (Figure 1.1) (68). Thus, ZIP4 likely functions to transport zinc from the intestinal lumen into the enterocyte while ZIP5 transports zinc from the bloodstream into the enterocyte (54).
Figure 1.1. Schematic representation of the distribution of various Zn\(^{2+}\) influx and efflux proteins throughout an enterocyte. This diagram was adapted from the work of Wang and Zhou (67). Structures of these proteins were obtained from the Protein Data Bank (‘MT’, 4MT2; ‘ZIP4’, 2CBN; other ZIPs, 2AJO; ‘ZnT1’, 3NWI; other ZnTs, 2ENK) and illustrated in MacPyMol Molecular Graphics System (Version 1.5.0.4 ©Schrödinger, LLC. 2010) (69-74). Where no structure was available, other protein structures from the same protein family were used.
1.4.2 Zinc Efflux

A number of studies have examined the mechanism of zinc efflux in an attempt to determine how cells remove excess zinc. A family of zinc efflux transporters, known as CDF, ZnT, or SLC30, depending on the organism, has been the focus of much investigation over the last 15 to 20 years. The most well-defined members of this protein family include the bacterial CDF (cation diffusion facilitator) and mammalian ZnT (SLC30) (75, 76). The name, ‘cation diffusion facilitator’ is deceiving; CDF proteins do not actually operate as diffusion facilitators but, instead, use existing gradients to drive the transport of zinc, similar to secondary active transporters (54). This family of metal ion transporters invariably transports zinc (and/or other metal ions) from the cytoplasm of the cell either into the lumen of intracellular organelles or into the extracellular space (Figure 1.1) (54).

Similar to a number of ZIPs, ZnT1 (SLC30A1) is thought to have a role in the absorption of dietary zinc and has been localized to the basolateral membrane of mammalian enterocytes (77, 78). ZnT1 is thus thought to efflux absorbed dietary zinc from the enterocyte into the bloodstream (77). Other ZnT proteins have been localized to various organelles or even organs, although their roles are less understood. For example, ZnT3 (SLC30A3) is confined to the brain and testis of rats, and ZnT2 (SLC30A2) and ZnT4 (SLC30A4) are expressed in rodent intestine, although they were expressed only in intracellular vesicle membranes (79, 80). From this observation, it seems plausible that ZnT2 is involved in the storage or excretion of zinc from enterocytes with high zinc influx (67). ZnT4 has also been localized to the trans-Golgi network and may promote zinc secretion into the bloodstream through exocytosis (81). ZnT6 (SLC30A6) is thought to form a complex with ZnT5 (SLC30A5) and activate zinc-dependent enzymes in the secretory pathway, although ZnT6 may also function to excrete zinc from enterocytes into the intestinal lumen through exocytosis (67, 82). ZnT7 (SLC30A7) is localized primarily to the Golgi apparatus and is thought to have a role in the incorporation of zinc into membrane-bound or secreted enzymes (82, 83).
1.4.3 *Zinc Sensing*

The concept of proteins that could ‘sense’ zinc levels significantly changed the understanding of zinc homeostasis. These zinc-sensing proteins are transcription factors that can alter the expression of zinc transporters and storage proteins based on the intracellular zinc concentration. The transcription factor, MTF1 was one of the first such zinc sensors to be characterized. MTF1, or metal response element-binding transcription factor 1, is a zinc finger protein that was found to induce transcription of metallothionein (zinc storage) when cells were treated with zinc (84). Little is known about zinc sensing proteins; the mechanism by which they ‘sense’ zinc levels and influence the transcription of zinc influx and efflux proteins has yet to be identified in most cases. Thus far, the known (and fairly well described) zinc sensing proteins include ZUR (a member of a family of uptake regulators), ZiaR (a repressor of a heavy metal transporter, ZiaA), ZnTR (a repressor of a zinc efflux transporter, ZnTA) and ZAP1 (a zinc influx regulator).

When bound to zinc, ZUR binds DNA and represses transcription of zinc uptake systems, including *znuABC, ycdH*, and *yciC* (1). ZUR is a member of a family of uptake regulators, along with FUR (an iron sensing transcriptional regulator), found in bacteria. Interestingly, FUR has a zinc-binding site and ZUR has an iron-binding site. In both cases, the zinc site is tetrahedral, utilizing two thiolate ligands and most likely a nitrogen from histidine, and the iron site is octahedral, known to utilize histidines and carboxylates (1). ZUR is thought to respond to zinc with a binding affinity of up to $10^{15} \text{M}^{-1}$ (50).

ZiaR is a zinc-responsive repressor of *ziaA*, which encodes an efflux transporter, ZiaA. ZiaR is a member of the ArsR family of metalloregulatory proteins which includes SmtB and ArsR (85). ZiaR contains one-high affinity zinc site, with a $K_F > 10^{11} \text{M}^{-1}$, which is thought to utilize at least one histidine and a cysteine (85).

ZnTR is a member of a family of MerR repressors. ZnTR, similar to ZUR, is thought to bind zinc with an affinity of up to $10^{15} \text{M}^{-1}$ and activate transcription of zinc efflux proteins, specifically the zinc efflux pump, ZnTA (50, 86). ZnTR functions as a dimer, binding two zinc ions per dimer, whereas ZnTA is a P-type ATPase transporter that consumes ATP to catalyze zinc export (86).
ZAP1 is a zinc-responsive transcriptional regulator that senses intracellular zinc levels and changes the expression of Zn$^{2+}$ binding proteins (87, 88). The mechanism of this regulation is essentially unknown, although ZAP1 (a zinc finger protein) has been shown to bind to the ZRE (the zinc responsive element) sequence upstream of a number of zinc-regulated genes such as the ZIP protein family and the ZRT proteins (87, 89). There is some evidence to suggest that ZAP1 may act as both a transcriptional activator and a repressor in the regulation of ZRT1 and ZRT2 (90).

Although there has been a significant amount of recent research delving into the mechanisms of zinc homeostasis, even the simplest prokaryotic systems have not been satisfactorily described because they are too complex. Most likely, many more proteins are involved in the transport of zinc throughout the cell than those that are currently known. Proteins with roles in the trafficking of zinc through cells, either from influx transporters to zinc requiring apo-enzymes or from influx to efflux transporters in intestinal cells, remain largely undefined. However, metallothionein is believed to have a role in the transport and storage of zinc, though the exact nature of this function is largely unknown. Metallothionein is the focus of this thesis, and is described in detail below.

1.5 Metallothionein

Initially isolated by Margoshes and Vallee in 1957 from horse kidney cortex (91), metallothioneins are a group of relatively small metalloproteins (approximately 4 to 8 kDa (92)) with a high cysteine content (~30% of the total amino acids (93)), but no disulfide bonds or aromatic amino acids (94-96). Metallothioneins were named for their unusually high metal (metallo) and sulfur (thiol) content. Members of the metallothionein (MT) family have been found to have cys-x-cys homology in organisms from bacteria (97) to fungi (98), to plants and mammals (99). Another unique characteristic of metallothioneins is the structure and metallation chemistry, which is dictated by the metal-thiolate bonds (100-102).

The best-characterized MTs are the mammalian MTs, which are composed of 60-80 amino acid residues (twenty of which are cysteines) and are able to coordinate multiple metal ions. The mammalian MT family is further subdivided into four subfamilies,
which are proposed to have specific functions in different tissues. Found in the liver and kidneys, MT 1 and MT 2 may be induced by metal ions, glucocorticoids and cytokines, as well as oxidative stress (103). MT 3 is primarily found in the brain, although it has also been detected in cells of the kidney, testes, and the tongue (104, 105). MT 4 is specifically expressed in stratified squamous epithelia, which are protective layers on the surface of tissues such as the skin, tail, and tongue in rodents (106, 107). The expression of MT 3 and MT 4 is more strictly controlled than that of MT 1 or MT 2. These differences in expression may be due to the potential detrimental effects if metal ion homeostasis is disrupted in the particular tissues where MT 3 and MT 4 are most often localized (108-110).

Many transition metals, specifically d^{10} metals, such as Zn^{2+} and Cu^{+}, are known to bind these unique sulfur-rich proteins in vivo. MT is believed to be important in copper and zinc homeostasis, but also aids in toxic metal detoxification and the reduction of oxidative stress. MT binds as strongly to toxic metals, such as Cd^{2+}, Hg^{2+}, and As^{3+}, as it does to essential metals, like Zn^{2+} and Cu^{+}, because of its twenty cysteiny1 thiols – following the Irving-Williams series (Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}) (111-113).

X-ray diffraction and NMR studies of rat liver MT 2 and rabbit liver MT 1, saturated with Zn^{2+} and Cd^{2+}, showed that the bound metal ions are arranged into two distinct metal-thiolate clustered binding domains using terminal and bridging cysteiny1 thiolate ligands (69, 114, 115). The N-terminal β-domain may bind up to three Zn^{2+}, three Cd^{2+} or six Cu^{+} using nine cysteines, while the C-terminal α-domain binds up to four Zn^{2+}, four Cd^{2+} or six Cu^{+} using eleven cysteines (Figure 1.2) (114, 116-118).

1.5.1 The Structure of Apo-Metallothionein
The majority of research has investigated the metallation properties of MT, specifically examining the structure of the saturated holo-protein. In vitro metallation studies begin by adding metals to either the apo-protein (119-121) or to the holo-protein (if bound to metals lower in the Irving-Williams series of affinities) (122, 123). For example, many metals can displace Zn^{2+} either isomorphously (e.g., Cd^{2+}; (124)) or by inducing a
Figure 1.2. The sequence, structure, and location of Cd$^{2+}$ binding in Cd$_7$-MT 1a. A space filling structure (A) and ball and stick models of the cadmium-cysteiny1-thiolate clusters (B) of Cd$_7$-βα-rhMT 1a are shown with the β-domain on the left and the α-domain on the right. (C) The sequence and metal connectivity diagram for MT 1a. Numbering of the Cd-thiolate centers is based on NMR studies by Messerle et al. (1990) (116). Adapted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
structural change (e.g., Cu$^+$; (125)). Metallation is expected to be completely different depending on the starting metallation state of the protein; metallation from the apo-protein is not the same as from the saturated holo-protein. There are benefits to using the holo-protein as the basis for these studies. Primarily, the metals protect the twenty cysteinyl thiols from oxidation (especially at near neutral, physiological pH where the sulfurs are particularly vulnerable). Additionally, toxic metal binding (e.g., Cd$^{2+}$) by liver metallothioneins in vivo would likely occur through the displacement of Zn$^{2+}$; this released Zn$^{2+}$ then induces the expression of de novo metallothionein.

Initial posttranslational metallation of metallothioneins, however, would begin with the nascent apo-protein. Although studying the apo structure may aid in understanding this key initial metallation step, determining the structure of the apo-peptide is complicated because it is highly fluxional and does not contain chromophorically useful residues. To circumvent these problems, molecular modelling was used to explore the apo-protein structure and suggested that the metal-free protein was loosely folded into a globular sphere with the thiols exposed on the surface of the protein (126-129). Any tertiary structure of MT is based on the metal coordination geometries; without metals, the apo-protein may still be described as a random-coil.

Recent electrospray ionization mass spectra provided novel experimental structural data for apo-MT (130). Similar to that predicted from molecular modelling (126-129), the charge state distribution of the apo-protein at physiological pH was more comparable with the Zn$_7$-protein than to the acid-denatured apo-protein at pH 3 (Figure 1.3). The main difference was that Zn$_7$MT appeared to have a slightly larger structure, which is attributable to a slight expansion of the globular structure to accommodate seven metal ions.

1.5.2 Metal-Induced Folding

Protein structure is intimately linked to protein function. For many metalloproteins, insertion of a metal ion cofactor is considered a post-translational modification and the metal ion is inserted after the protein folds to accommodate it (131, 132). For other metalloproteins, the metal is required for proper protein folding (131). To date, little is
Figure 1.3. Charge state mass spectra and molecular models of apo- and holo-MT. Charge state spectra and molecular models of denatured (pH 3) apo-βα-rhMT 1a (A), globular (pH 7) apo-βα-rhMT 1a (B), two-domain holo-βα-rhMT 1a (C), and βα-rhMT 1a bound to twenty benzoquinone cysteiny1 modifiers (D). Adapted from Biochemical and Biophysical Research Communications, 425, Summers, K. L., Mahrok, A. K., Dryden, M. D. M., and Stillman, M. J., Structural properties of metal-free apometallothioneins, 485-492. © 2012 with permission from Elsevier.
known about metalloprotein assembly and metal ion insertion, although there is much research being conducted on the subject (8).

Azurin, an electron transferring blue-copper protein, does not require a metal ion for folding, although protein folding is much faster when copper is present (8, 133). Because there are essentially no free copper ions in the cytoplasm (due to the oxidative damage to proteins, lipids, and nucleic acids that would result), copper is thought to be inserted into the folded protein by a copper chaperone in vivo (8). Conversely, many zinc finger proteins require Zn$^{2+}$ binding to fold; metal coordination is thought to induce secondary and tertiary structure formation (8).

Metallothionein is similar to the zinc finger proteins in that metal binding induces protein folding. Although MT is described as a protein that undergoes folding, it does not have defined secondary structures such as the α-helix and β-sheet present in the zinc finger motif. In MT, the covalent metal-sulfur bonds define the structural properties. Unlike proteins requiring a single metal ion cofactor, there is ambiguity in the position of metals in partially metallated MT intermediates – in terms of metal coordination into specific binding sites. The metal-binding-induced structure of MT is believed to require translation of the entire polypeptide chain before metallation and folding can occur (126).

### 1.5.3 Structure of Holo-Metallothionein

In the more than 50 years since the discovery of equine kidney MT, only two crystal structures have successfully been obtained for the mammalian branch of this metalloprotein family. The first structure, published in 1986 by Furey et al. (134), was of rat liver MT 2 bound to five Cd$^{2+}$ and two Zn$^{2+}$; this structure was later refined by Robbins, et al. in 1991 (114). In 2005, a structure of the yeast Cu$_8$-MT was reported by Calderone, et al. (135). These crystal structures provided important information on the coordination of Cd$^{2+}$ and Zn$^{2+}$ (tetrahedral), and Cu$^+$ (trigonal or digonal or a combination thereof) to the cysteinyl sulfur ligands. Unlike the solution structures proposed by $^{115}$Cd-NMR (115, 136), $^1$H-NMR (102, 117, 136), XAFS (137, 138), and molecular modelling (139), the crystal structures provided information about the domain linkage in addition to information about the metal-binding sites and the individual domains. The structure of
MT was described as a dumb-bell-like shape with two clustered metal binding domains separated by a linker region (69, 114). The holo-structure is rigid, defined by metal ion coordination, which can include digonal, trigonal, or tetrahedral geometries, or possibly some combination (94). The stable saturated clusters resulting from complete saturation with metal ions, are often characterized by the stoichiometric ratio of metal ions to protein (usually from seven to eighteen metal ions per mammalian MT protein), which depends on the metal and its preferred coordination (140).

Coordination of Zn$^{2+}$ or Cd$^{2+}$ was found to require both terminal (one cysteinyll sulfur bound to one M$^{2+}$) and bridging (one cysteinyll sulfur bound to two M$^{2+}$) cysteines in each domain (Figure 1.2). In the N-terminal β-domain, six terminal and three bridging cysteines bind three M$^{2+}$; and in the C-terminal α-domain, six terminal and five bridging cysteines bind four M$^{2+}$. The two metal-thiolate clusters are believed to be arranged in a way that allows the binding of multiple different metal ions in each domain. The two-domain structure is thus thought to aid in the maintenance of both Zn$^{2+}$ and Cu$^+$ homeostasis simultaneously (118, 141). In contrast to the X-ray structure (of rat liver Cd$_5$Zn$_2$-MT; (69)), which depicts the characteristic dumb-bell shape, molecular models of holo-MT suggest the domains may actually coalesce in solution (126).

1.5.4 Putative Functions

The putative functions of MT include toxic metal sequestration (e.g., Cd$^{2+}$, Hg$^{2+}$, As$^{3+}$), protection from oxidative stress, and essential metal trafficking and storage (e.g., Cu$^+$ and Zn$^{2+}$). MT may bind to numerous ‘soft’ transition metals (and even some metalloids) (e.g., Zn$^{2+}$ (93); Cd$^{2+}$ (93, 142); Hg$^{2+}$ (143); Cu$^+$ (144, 145); Ag$^+$ (144); Au$^+$ (144); Pt$^{2+}$ (144); As$^{3+}$ (111); Bi$^{3+}$ (146); and Tc$^{5+}$ (147)) because of its many thiols, but many of these binding events have been demonstrated in vitro only and the biological relevance is largely unknown. However, in studies examining the role of MT in metal detoxification, particularly utilizing MT knockout organisms, the resistance to toxic metals diminishes without MT. MT 1 and MT 2 knockout mice developed normally until exposed to cadmium, at which point the mice died of hepatic damage, providing concrete evidence that MT is important in reducing the toxic effects of cadmium exposure (148).
Metallothionein may not only sequester toxic metals, but may also prevent the toxic effects of essential metals. For example, MT may aid in the prevention of copper toxicity simply by reducing or sequestering it (149). In particular, MT was reported to be beneficial in alleviating the symptoms of both Menkes and Wilson’s disease. In Menkes disease, copper accumulates in the kidneys and the intestinal wall, depriving the brain, serum and liver of adequate copper (150). Using a mouse model of Menkes disease, in which the MT 1 and MT 2 genes were inactivated, the mice were found to be more susceptible to copper toxicity without inducible MT (150, 151). In Wilson’s disease, copper accumulates in the liver, brain, and cornea, leading to cellular damage and the eventual release of free copper into the blood (152). In studies on hepatocytes from patients with Wilson’s disease, accumulation of Cu-MT instead of free copper ions was found to lessen the cellular damage slightly (153).

MT is thought to have an important function as a protective agent against oxidative stress. In fact, a redox cycle involving MT has been proposed in explanation of how MT might fulfill this role within a cell (154). Reactive oxygen species oxidize MT, causing the formation of disulfide bonds and the release of Zn\(^{2+}\). The released Zn\(^{2+}\) then up-regulates Zn\(^{2+}\) binding proteins through MTF-1 binding. Subsequent reduction of the cysteinyi sulfurs by glutathione, or synthesis of de novo MT, then re-establishes the initial zinc balance.

MT has also been implicated in the trafficking and storage of essential metals like Cu\(^{+}\) and Zn\(^{2+}\). Zn\(_7\)-MT has been shown to act as both a Zn\(^{2+}\) donor for apo-enzymes, such as m-aconitase (155), carbonic anhydrase (156, 157) and Gal4 (158) and a Zn\(^{2+}\) acceptor in the transfer of Zn\(^{2+}\) from Sp1 (a zinc finger protein) to MT (159). The presence of partially metallated species has been noted during the donation of Zn\(^{2+}\) from Zn\(_7\)MT to metal ion chelators or to apo-enzymes (160-162), which could suggest that partially metallated MT also functions in regulating intracellular Zn\(^{2+}\) concentrations. Interestingly, a recent study has demonstrated that Zn\(_7\)-MT 3 can exchange its bound Zn\(^{2+}\) with the Cu\(^{2+}\) bound to the amyloid-β-peptide, resulting in the formation of Cu\(_4\)Zn\(_4\)-MT 3 without the threat of cysteinyi thiol oxidation (109).
Even after 50 years of research on the structure and function of MT, a few gaps remain in our understanding. An explanation of how Zn\textsubscript{7}MT – with stronger binding affinities than recipient apo-enzymes – donates Zn\textsuperscript{2+} is missing in the literature. As discussed above, apo-MT is very flexible, without significant secondary structure (130); holo-MT is rigid, dominated by the coordination geometry of the bound metal ions (94). Working with the current understanding of how metallothionein binds and stores metals, donation of Zn\textsuperscript{2+} from the two-domain Zn\textsubscript{7}MT would cause the clustered binding sites to unfold and would most likely lead to the exposure and subsequent oxidation of the cysteinyl thiols (154, 155, 157, 158).

1.6 Electrospray Ionization Mass Spectrometry

ESI is often referred to as a 'soft ionization' technique because there is very little fragmentation during the ionization process, which can be advantageous for biological samples that are defined by non-covalent interactions (e.g., metal binding to amino acids) (163, 164). Particularly in the study of metallothioneins, ESI MS has permitted a much better understanding of the metallation states of the protein. Because MT has no true defined secondary structure, using spectroscopic techniques to study MT – especially the apo-protein – is quite challenging (100-102). Additionally, the main metal ion stored by mammalian MT, Zn\textsuperscript{2+}, is spectroscopically silent (165). Consequently, a zinc substitute is required to utilize many of the techniques typically used to study metalloproteins (e.g., UV absorption, CD and NMR spectroscopies). Cadmium has been used extensively for this purpose because, structurally, cadmium coordination to thiols is essentially the same as zinc and because, not surprisingly, Cd-MT and Zn-MT appear almost identical in NMR structural studies (116, 117). The main benefit to studying cadmium, as compared to zinc, is that the ligand-to-metal charge transfer associated with cadmium-thiolate bonds is red shifted (to approx. 250 nm) compared to zinc-thiolate bonds (at approx. 230 nm) and therefore Cd-MT, unlike Zn-MT, can be monitored using both UV absorption and CD spectroscopies (142). Cadmium also has a greater affinity for thiolate groups, which provides increased protection for cadmium-saturated MT against oxidation (93, 166, 167).
Although cadmium has been very effective in the elucidation of some aspects of zinc binding in metallothionein, ESI MS has made the direct analysis of Zn-MT feasible (168-170). Electrospray ionization mass spectrometry permits the determination of the mass of the metal ions (and therefore allowing one to deduce which metal is bound) as well as the number of metal ions bound to the protein (by analyzing the increase in the charge state masses as compared to those of the apo-protein). Conversely, spectroscopic techniques are often capable of providing only an average of the total metals bound. Prior to the more prevalent use of ESI MS to study metal-binding reactions, solutions of MT were often assumed to contain only one metallated species (i.e., if four equivalents of Cd\(^{2+}\) were added, this would result in the formation of 100% Cd\(_4\)-MT); however, it is now obvious that the addition of metal ions to solutions of MT results in a distribution of metallated species (120). To date, ESI MS has provided a large amount of information on the partially metallated intermediates in the MT metallation pathways of both essential (e.g., Zn\(^{2+}\) (161) and Cu\(^{+}\) (171, 172)) and toxic metals (e.g., Ag\(^{+}\) (172), As\(^{3+}\) (111, 173-175), Cd\(^{2+}\) (120, 176, 177), and Bi\(^{3+}\) (146)).

### 1.6.1 Metal Binding Studies

When observed by ESI MS, binding of metal ions to an apo-protein causes an increase in the mass to charge ratio (m/z) that is proportional to the mass of the metal ion bound. From the deconvoluted spectra, the stoichiometry of metals bound to the protein can easily be determined because the difference in mass between the apo- and holo-proteins should be some multiple of the metal ion of interest. Similarly, the relative distribution of metallated species in a solution – in proteins like MT that bind multiple metal ions – can now be monitored easily. Many of the metal binding studies conducted using ESI MS are in fact on metallothioneins. ESI MS has been utilized in many metal-binding studies of metallothioneins from a variety of organisms with a number of metals including Cd\(^{2+}\) (92, 120, 176, 177), Zn\(^{2+}\) (92, 161), Cu\(^{+}\) (92, 121, 178), Ag\(^{+}\) (172), Bi\(^{3+}\) (146), and As\(^{3+}\) (111, 174, 175).

ESI MS techniques have also been employed in the study of other metal binding proteins. For example, the calcium binding of parvalbumin and calmodulin has been investigated by ESI MS (179). Cooperative binding in the two calcium-binding sites of parvalbumin
was observed, and up to four Ca\textsuperscript{2+} were found to bind to calmodulin, with the last two sites binding metal ions cooperatively (179). In another study, human insulin growth factor 1 was found to bind four Cu\textsuperscript{+} ions cooperatively, possibly into a tetracopper-hexathiolate cluster (i.e., Cu\textsubscript{4}S\textsubscript{6}) using ESI MS (180). In yet another ESI MS study, the *Escherichia coli* protein SlyD, was found to bind up to seven Ni\textsuperscript{2+} non-cooperatively and with sub-micromolar binding affinity (181).

ESI MS may also be used in structural studies of proteins, like metallothioneins, that exhibit metal-induced folding. For example, ESI MS was used to show that binding of the first Zn\textsuperscript{2+} to a vitamin D receptor results only in a slight conformational change, but binding of a second Zn\textsuperscript{2+} results in a significant alteration in protein structure (182).

### 1.7 Scope of Thesis

The purpose of the research summarized in this thesis was to characterize the zinc metallation of human metallothionein 1a using ESI MS. Initially, the metallation of each individual domain peptide, and the full two-domain protein (Section 3.1), was examined to determine if the protein exhibited any cooperativity in Zn\textsuperscript{2+} binding, as had been proposed previously. Metallation of human metallothionein with Zn\textsuperscript{2+} was hypothesized to occur non-cooperatively, similar to metallation with Cd\textsuperscript{2+}, but in contrast to established theories.

The first competition experiment (Subsection 3.2.1), in which the individual domains compete for available Zn\textsuperscript{2+}, was intended to determine if either domain bound the added Zn\textsuperscript{2+} with a higher affinity. If this were the case, it could provide evidence for a domain-specific metallation mechanism where one domain was saturated prior to the filling of the second domain. It was hypothesized that the α-domain would have a higher Zn\textsuperscript{2+} binding affinity than the β-domain based on previous work and because the α-domain simply has more binding sites than the β-domain (183, 184). However, some previous reports have also suggested that the β-domain binds metals preferentially (145).

The second competition experiment (Subsection 3.2.2), in which the domain peptides compete with the full protein for binding of the available zinc, was originally designed to
investigate the role of the inter-domain, amino acid linker on Zn\(^{2+}\) binding. It was hypothesized that the connected domains (i.e., the full two-domain protein) would bind Zn\(^{2+}\) much more strongly than the separate domains, thus explaining the inherent evolutionary advantage of a two-domain structure.

Because ESI MS is able to distinguish between each individual metallated species, it proved to be a much more useful tool than others that detect an average of the total species in solution. Through the use of this powerful tool, coupled with data simulations, the Zn\(^{2+}\) binding mechanism for human MT 1a was elucidated. With this knowledge in hand, and detailed molecular modelling and molecular dynamics calculations, a novel metallation pathway for human MT 1a was proposed.
2 Methodology

The experiments described in this thesis utilized a recombinant version of human MT 1a purified from an *Escherichia coli* expression system. Recombinant proteins are often preferred over purification of proteins from live mammals. In addition to the more obvious concerns around caring for live animals and later euthanizing them, the many isoforms of mammalian metallothionein cannot be readily separated. This may be problematic because the different isoforms are known to have distinct roles and, most importantly for studies using mass spectrometry, the isoforms have different masses.

The study of human MT in particular could not be studied in depth without the expression of recombinant proteins in a host organism. Recombinant protein expression simplifies protein isolation and purification, resulting in large quantities of one MT isoform. The use of a recombinant expression system also permits the design and production of truncated versions of proteins. This attribute is particularly important to the studies detailed herein because it permits the expression and purification of the two domains of MT as individual peptides (β- and α-rhMT).

2.2 Protein Preparation

The expression and purification methods used to obtain human recombinant MT isoform 1a have been previously reported (185, 186). The details of the purification process are provided below; in essence, recombinant human MT 1a was expressed in *E. coli*, purified using both ion exchange and size exclusion chromatography, and the purity of the protein assessed using UV absorption spectroscopy, before experimentation using ESI MS.

2.2.1 Recombinant Human Metallothionein 1a Constructs

The plasmid vectors for the recombinant peptides of human metallothionein isoform 1a were constructed using the pET29a plasmid (Novagen/EMD Biosciences, San Diego, USA) at Cardiff University, Wales, UK and transformed into competent *E. coli* BL21 (DE3) *pLysS* cells (Novagen/EMD Biosciences) at the University of Western Ontario, London, Canada (187, 188). The pET29a plasmid contains a kanamycin resistance gene to select for cells that contain the plasmid with the recombinant protein sequence. The
sequences of the DNA constructs were based on the sequence of the native protein, but do contain minor sequence variations from that published by Richards et al. (189). The recombinant β-rhMT, α-rhMT and βα-rhMT 1a proteins are composed of 38-residue, 41-residue, and 72-residue sequences, respectively: β-rhMT MGKAAAAACSC ATGGSCTCTG SCKCCKECKCN SCKKAAAA, α-rhMT MGKAAAAAC CSCCPMSCAK CAQGCVCKGA SEKSCCKKA AAA, βα-rhMT MGKAAAAACSC ATGGSCTCTG SCKCCKECKCN SCKKAAAAACC SCCPMSCAKC AQGCVCKGAS EKCSCCKKAA AA. The expression system includes an N-terminal S-peptide tag on the N-terminus of each protein (MKETAAAKFE RQHMDSPDLG TLVPRGS), which was originally used as an affinity tag for purification, but is now retained simply for stability purposes. The transformed cells were grown in LB broth with cadmium and kanamycin until the absorbance at 600 nm was approximately 0.6. The cells were then re-suspended in 1 mL solutions of 50% (v/v) LB medium and 50% (v/v) glycerol and stored long-term at -80 °C (188).

2.2.2 Protein Expression

A portion of the glycerol stock solutions of transformed E. coli cells was streaked on LB agar containing 50 µg/mL of kanamycin and 50 µM of CdSO₄ (Fisher Scientific) and incubated at 37°C overnight. Large preparations (4 × 1 L) were produced by inoculating the transformed cells from the agar plates equally into four Fernbach flasks containing 1 L of LB liquid cell culture broth with 50 µg/mL kanamycin and 50 µM CdSO₄. Cadmium was added to both the agar plates and the initial liquid culture to protect any MT expressed at a basal level during cell growth. The culture flasks were stirred at 175 rpm on a cell shaker incubated at 37°C to induce rapid growth of the E. coli cells until the solution reached an absorbance of approximately 0.6 at 600 nm (Varian Cary® 500). At an OD₆₀₀ of 0.6, the E. coli cells are believed to be in the log-phase of their growth cycle. Protein expression was then induced by the addition of 700 µM (final concentration) isopropyl-β-D-thiogalactoside (IPTG) and the CdSO₄ concentration was increased to 250 µM to stabilize the nascent MT. Incubation and shaking continued for four hours, at which point the cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C
re-suspended in 35 mL of 10 mM tris(hydroxymethyl)aminomethane (Tris) at pH 7.4, and stored at -80°C.

2.2.3 Protein Purification

Semi-thawed cells were lysed using a cell disruptor (Constant Systems Ltd.) and centrifuged at 13 000 rpm and 4°C for 1 h (Avanti J-series centrifuge; JA-25.50 rotor; Beckman-Coulter) to separate the soluble proteins in the supernatant from the cellular debris. After isolation of soluble proteins, all manipulations of the protein solutions were completed on ice and with minimal oxygen exposure. The protein solution was gravity filtered and rhMT 1a was isolated by tandem cation exchange columns (HiTrap™ SP Sepharose™ HP cartridges; Amersham Biosciences/GE Healthcare) coupled to an LC pump (Dionex/ Thermo Scientific). The two buffers required for the ion exchange column were 10 mM Tris-HCl (pH 7.4; washing buffer) and 10 mM Tris-HCl with 1 M NaCl (pH 7.4; elution buffer). Eluent from the column passed through a quartz flow-cell and was monitored by UV absorption spectroscopy (Varian Cary® 50, Agilent Technologies) using the Cary® WinUV Scanning Kinetics software to scan continuously over the 200-300 nm region. The washing buffer was used to remove any molecules that adhered weakly to the column; the column was washed with approximately fifteen column volumes (~150 mL) at 2.5 mL/min or until the absorbance returned to the baseline. The elution buffer was used to elute the bound MT, again at a flow rate of 2.5 mL/min, using a gradient of increasing salt concentration up to 25% (v/v) elution buffer and 75% (v/v) washing buffer.

Fractions with the distinctive UV absorption at 250 nm (corresponding to the ligand-to-metal charge transfer transition of the S-Cd bonds in the metal-thiolate clusters) (Figure 2.1), which were assumed to contain rhMT 1a, were pooled and concentrated to approximately 10 mL using a stirred Ultrafiltration Cell with a 1000 Da cut-off membrane (Amicon Bioseparations/ Millipore) and pressure from a nitrogen gas line.
Figure 2.1. The UV absorption spectra of apo-rhMT 1a and Cd₇-rhMT 1a. The apo-protein is shown in green and the holo-protein is shown in blue.
2.2.4 Cleavage of the N-terminal S-tag

Removal of the S-tag was achieved using a thrombin enzyme (Thrombin CleanCleave™ Kit, Sigma). Thrombin is a serine protease that cleaves polypeptides at a specific recognition sequence (X-X-Pro-Arg/Lys•Y-Y, where X is a hydrophobic residue, Y is a non-acidic residue, and the • represents the bond that is cleaved). For the recombinant MT fusion protein detailed above, the sequence recognized by the cleavage enzyme is Leu-Val-Pro-Arg•Gly-Ser. In the kit, the thrombin is immobilized on agarose beads and stored at -20°C as a mixture of 50% (v/v) glycerol and 50% (v/v) 20 mM Tris-HCl (pH 8.0; storage solution). The resin was washed in a solution of 50% (v/v) 50 mM Tris-HCl (pH 8.0) and 50% (v/v) 10 mM CaCl₂ (1× cleavage buffer) and centrifuged at 2500 rpm for 5 min (Eppendorf centrifuge 5418). The supernatant was removed and the thrombin-coated beads were re-suspended in 90% (v/v) 500 mM Tris-HCl (pH 8.0) and 10% (v/v) 100 mM CaCl₂ (10× cleavage buffer). The concentrated protein solution was added to the thrombin beads under argon gas and stirred constantly overnight at 4°C. The thrombin beads were then recovered from the protein solution by centrifugation, washed in 1× cleavage buffer and an equal volume of 50 mM Tris-HCl (pH 8) with 500 mM NaCl, and re-suspended in the storage solution for preservation at -20°C.

The supernatant containing the cleaved protein was collected and desalted using a size exclusion column (Sephadex™ G-25) pre-equilibrated with a 10 mM Tris buffer (pH 7.4), and the UV absorbance between 200 and 300 nm. The recombinant β-MT, α-MT and βα-MT protein fractions were separated from the S-tag using cation exchange with the same washing and elution buffers as above, and concentrated to approximately 10 mL using the Ultrafiltration Cell system. The S-tag peptide bound weakly to the cation exchange column and eluted during washing; the recombinant β-MT, α-MT and βα-MT proteins eluted from the column with approximately 15% (v/v) elution buffer.

At this point, the purified Cd₃-β-rhMT, Cd₄-α-rhMT and Cd₇-βα-rhMT 1a protein solutions were separated into 1 mL fractions and stored at -20°C until experimentation with ESI MS. Rigorous evacuation followed by argon saturation of all buffers, reagents, and protein solutions was used to impede oxidation of the cysteine residues. Low temperatures were used to aid in preventing protein degradation.
2.2.5 Preparation of Apo-Metallothionein

Apo-metallothionein was prepared by adding 200 µL concentrated HCl to a thawed 1 mL sample of purified Cd_{3}-β-rhMT, Cd_{4}-α-rhMT or Cd_{7}-βα-rhMT 1a to demetallate the protein, followed by buffer exchange on a size exclusion column (Sephadex™ G-25) at 4°C using formic acid at pH 2.7 (in >16 MΩ cm deionized water; Barnstead Nanopure Infinity). Low pH conditions cause denaturation and loss of the metal ions bound to MT, and size exclusion chromatography separates the metals from the protein. This method simultaneously demetallates and desalts the solution. Because MT is devoid of aromatic amino acids, the metal-free protein fractions were detected using UV absorption at 220 nm (corresponds to electronic transitions from the polypeptide backbone) (Figure 2.1). A formic acid buffer (pH 2.7) was chosen for analysis to aid in the stability of the metal-free protein and slow down oxidation of the many cysteine residues, as well as ensure MS compatibility.

2.2.6 Protein Quantification

Protein concentrations were determined by re-metallation of a portion of the apo-rhMT with Cd^{2+}. The concentration of Cd-rhMT was then determined by UV absorption spectroscopy using absorbance at 250 nm, which corresponds to the ligand-to-metal charge transfer transition generated by cadmium-bound MT (Ɛ_{β} = 36 000 M^{-1}cm^{-1} (186, 190); Ɛ_{α} = 45 000 M^{-1}cm^{-1} (186); Ɛ_{βα} = 89 000 M^{-1}cm^{-1} (186, 191)).

2.3 ESI MS Procedures

2.3.1 Solution Conditions

Protein solutions were prepared in formic acid at pH 2.7 (in <16 MΩ cm deionized water). Final ESI MS solutions were adjusted to biological pH (approximately pH 7.4) using concentrated ammonium hydroxide. The solutions were thoroughly de-aerated by evacuation and saturated with argon to remove the majority of the oxygen and to prevent oxidation of the apo-protein when the pH was raised. All protein solutions remained sealed and on ice during experimentation to help prevent oxidation and protein degradation.
2.3.2 Instrument Settings and Calibration

Mass spectra were collected on an electrospray ionization time-of-flight mass spectrometer (micOTOF-II, Bruker Daltonics) in the positive ion mode. NaI was used as the mass calibrant. The scan conditions for the spectrometer were as follows: end plate offset -500V; capillary +4200 V; nebulizer 2.0 bar; dry gas flow 8.0 L/min; dry temperature 80°C; capillary exit 180 V; skimmer 1 22.0 V; hexapole 1 22.5 V; hexapole RF 600 Vpp; skimmer 2 22 V; lens 1 transfer 88 µs; and lens 1 pre pulse storage 23 µs. The range was 500.0 - 3000.0 m/z, averaging 2 × 0.5 Hz. Spectra were collected and deconvoluted using the Bruker Compass Data Analysis software package.

2.3.3 Metallation with Zn\textsuperscript{2+}

Zinc sulfate (10 mM; Caledon Laboratory Chemicals) was prepared in <16 MΩ cm deionized water; all molar equivalents were determined using atomic absorption spectrometry (Varian AA240). For competition experiments, protein solutions were prepared such that all proteins in a sample were present in equimolar concentrations. Spectra of apo-proteins were recorded to ensure that the expected masses were observed within a small amount of error (±0.1%). Increasing amounts of Zn\textsuperscript{2+} were then added to protein solutions in molar equivalents using a syringe to inject the ZnSO\textsubscript{4} solution through the septum cap and lessen the exposure to oxygen.

2.4 Molecular Modelling

Molecular mechanics (MM3) and molecular dynamics (MD) calculations are often used in protein studies to visualize or predict the folding of a protein, the relative locations of the amino acids to one another, and/or the movement of the protein in solution (192).

Modelling parameters and sequence information have been previously described for studies of rhMT 1a (126). MM3 and MD calculations, parameterized using the modified force field described by Chan et al. (193) and using the dielectric constant for water (78.4), were carried out to obtain the minimum-energy structures of apo-βα-rhMT 1a, and Zn\textsubscript{n}-βα-rhMT 1a (where n = 1 to 7) (Subsection 3.2.2, Figure 3.16). The original Cd\textsubscript{7}-βα-rhMT 1a was modified to produce molecular models for the intermediate species in the
metallation reactions with Zn$^{2+}$ (185). Molecular modelling calculations, using the same parameters referenced above, were conducted using Scigress (Version 3.0.0, Fujitsu Poland Ltd.) operating on Intel® Core™ i7 PCs with 8 GB RAM. Each structure was energy minimized using the MM3 calculation followed by the MD simulation at 300 K for 5000 ps.
3 Results and Discussion

3.2 Non-Cooperative Zn\textsuperscript{2+} Binding

Since mammalian metallothionein (MT) was initially isolated in 1957 (91), the exact mechanism of the metallation of MT has remained a mystery. The established hypothesis, mainly developed from NMR spectroscopy, is that MT metallates in a cooperative ‘all or nothing’ fashion (183). For proteins that bind their substrates cooperatively, the only considerable species are the substrate-free or fully saturated protein because intermediates would likely be too unstable to take part in cellular chemistry.

Metallation of MT has been proposed to occur via a domain-specific cooperative mechanism, where the first four Cd\textsuperscript{2+} are exclusively bound by the α-domain, and the following three Cd\textsuperscript{2+} are bound by the β-domain. In this cooperative mechanism of metallation, the only stable species that are expected to be observed are the apo- and holo-protein, and possibly a Cd\textsubscript{4}-species. The apo-protein, with twenty free cysteine thiols, would be highly susceptible to oxidation and any partially metallated forms would likely be unstable and almost as vulnerable to oxidation.

A number of acid-induced demetallation studies of MT have suggested that demetallation of the two clustered binding domains occurs as a two-step cooperative process in which each individual step results in the complete loss of one of the clusters (177, 185, 194, 195). This model suggests that acid-induced loss of a single metal ion destabilizes the cluster and promotes complete metal ion dissociation. In contrast to this theory are studies that suggest that holo-MT is capable of donating several of its metal ions to Zn\textsuperscript{2+} depleted apo-enzymes without complete demetallation (196). In addition, oxidizing agents have been shown to release some of the Zn\textsuperscript{2+} bound to MT (197).

Understanding the correct mechanism of MT metallation profoundly impacts our understanding of the stability of the clustered holo-protein, as well as any partially metallated species, and their role in cellular chemistry; however, delineation of the mechanism depends entirely on the ability to distinguish partially metallated
intermediates. To further investigate the mechanism of metallation, Stillman and colleagues have previously used the discriminating power of ESI MS to demonstrate the stability of partially metallated forms of MT 1 with Cu\(^+\) (172, 178), Ag\(^+\) (172), Cd\(^{2+}\) (120, 176), As\(^{3+}\) (111, 173), and Bi\(^{3+}\) (146). ESI MS measures the mass-to-charge ratio of all species in solution; deconvolution of the mass spectra provides the stoichiometric ratios of the various metallated species. Stable partially metallated MT was observed during ESI MS metallation experiments with Cd\(^{2+}\), Bi\(^{3+}\), and As\(^{3+}\), supporting a non-cooperative mechanism for binding of these metals (111, 120, 146, 173, 175, 176, 198).

Many proteins do not show any form of cooperativity in substrate binding. In these proteins, substrate-binding events are independent and are described as non-cooperative. If Zn\(^{2+}\) binding to MT is non-cooperative, the K\(_F\) is expected to depend on the number of available binding sites and therefore, the binding affinity is expected to decrease statistically with each successive binding event (140, 199). In this case, with limiting amounts of metal ions, not all metal binding sites would be occupied and a number of partially metallated species are expected. The kinetic data of Ngu et al. (111, 173, 174, 200) provided clear evidence for non-cooperative metallation with As\(^{3+}\); the series of rate constants determined from time-dependent As-metallation diminished for each successive metal bound up to the sixth, and final, As\(^{3+}\).

### 3.2.1 Metallation of the β-domain with Zn\(^{2+}\)

With confirmation that metallation of MT with Cd\(^{2+}\) and As\(^{3+}\) follows a non-cooperative mechanism it is important to establish the binding mechanism for essential metals, such as Zn\(^{2+}\) and Cu\(^{+}\). A clearer understanding of the metallation mechanism of MT with Zn\(^{2+}\) and Cu\(^{+}\) will allow for a much better understanding of the role MT plays as a metallochaperone in the homeostasis of these metals.

The mass spectral data recorded during the metallation of apo-β-rhMT with Zn\(^{2+}\) clearly show that the stepwise Zn\(^{2+}\) metallation of apo-β-rhMT leads to the formation of partially metallated species (Zn\(_1\)- and Zn\(_2\)-β-rhMT) at sub-stoichiometric equivalents (< 3) of Zn\(^{2+}\) (Figure 3.1). Complete metallation occurs when approximately 3.5 equivalents of Zn\(^{2+}\) per β-rhMT were added to the solution. Complete metallation was expected to occur
Figure 3.1. Mass spectra recorded during the titration of apo-β-rhMT 1a with Zn$^{2+}$. Spectra were recorded as aliquots of 7.3 mM ZnSO$_4$ were added to a 29.2 µM solution of apo-β-rhMT 1a (pH 9.2). (A-E) Charge state data (left) and deconvoluted data (right) were recorded at molar equivalents of 0.1, 1.7, 2.6, 3.3, and 3.5 of Zn$^{2+}$. Metallation in excess of Zn$_3$-β-rhMT is a result of non-specific adducts. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
after exactly 3 Zn$^{2+}$ were added to β-rhMT; however, the discrepancy in the equivalents needed for complete metallation may be attributed to slight errors in the estimation of the initial metal-free protein concentration, as well as to the formation of low concentrations of Zn(OH)$_2$.

It is clear that the most abundant metallated species is a direct result of the number of equivalents of Zn$^{2+}$ added to the solution. When 1.7 stoichiometric equivalents of Zn$^{2+}$ are added to apo-β-rhMT, the dominant species is Zn$_1$-β-rhMT (Figure 3.1 B), while at 2.6 equivalents of Zn$^{2+}$ the two most abundant species are partially metallated Zn$_2$-β-rhMT and fully metallated Zn$_3$-β-rhMT (Figure 3.1 C). The deconvoluted spectra can be used as approximations of the actual metal ion distribution in solution because the ionization potential of MT, and its isolated domains, is similar for all metallation states (from apo-MT to fully metallated MT) (111, 173, 174, 200).

The relative abundance of each metallated β-rhMT species clearly demonstrates that partial metallation occurs and therefore provides support for the hypothesis that metallation occurs in a non-cooperative manner – at least for the N-terminal domain. At each equivalent of Zn$^{2+}$, with the exception of the beginning and end of the titration, the β-rhMT exists as a mixture of metallated states. At low equivalents of Zn$^{2+}$ partially metallated species are most abundant (although the apo-protein is also present), while at higher equivalents of Zn$^{2+}$ the fully metallated β-rhMT is most abundant. The linear progression of the metallation from apo-β-rhMT to Zn$_3$-β-rhMT further supports a non-cooperative mechanism, where the declining affinity of Zn$^{2+}$ for β-rhMT is directly related to the number of available metal-binding sites (Figure 3.2).

### 3.2.2 Metallation of the α-domain with Zn$^{2+}$

From the mass spectra recorded during the stepwise metallation of apo-α-rhMT with Zn$^{2+}$, it is evident that metallation leads to the formation of partially metallated species (Zn$_1$-, Zn$_2$-, and Zn$_3$-α-rhMT) at sub-stoichiometric equivalents (< 4) of Zn$^{2+}$ (Figure 3.3), similar to Zn$^{2+}$ metallation of the β-domain. The dominant metallation state is again a direct result of the number of equivalents of Zn$^{2+}$ added to solution. When 2.2 stoichiometric equivalents of Zn$^{2+}$ are added to apo-α-rhMT, the dominant species is Zn$_1$-
**Figure 3.2** Contour and bar graphs of the metallation of β-rhMT 1a as a function of Zn$^{2+}$ added. (A) A top-down view of metallation, showing the linear progression from apo-β-rhMT to Zn$_3$-β-rhMT as a function of Zn$^{2+}$ added to the solution (100% abundance is shown in red and 0% abundance is dark blue). (B) A bar graph of metallation, showing the relative abundance of each species as a function of Zn$^{2+}$ added to the solution. Colour code: apo-β-rhMT is red; Zn$_1$-β-rhMT is green; Zn$_2$-β-rhMT is dark blue; Zn$_3$-β-rhMT is light blue. Plots are based upon the deconvoluted mass spectra in Figure 3.1. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
Figure 3.3. Mass spectra recorded during the titration of apo-α-rhMT 1a with Zn$^{2+}$. Spectra were recorded as aliquots of 7.3 mM ZnSO$_4$ were titrated into a 34.1 µM solution of apo-α-rhMT 1a (pH 9.5). (A-E) Charge state spectra (left) and deconvoluted spectra (right) of α-rhMT were recorded at Zn$^{2+}$ molar equivalents of 0.4, 2.2, 3.7, 3.9, and 4.1. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
α-rhMT (Figure 3.3 B), while at 3.7 and 3.9 equivalents of Zn$^{2+}$ the dominant species are Zn$_2$- and Zn$_3$-α-rhMT, respectively (Figure 3.3 C and D). Complete metallation is observed at 4.1 equivalents of Zn$^{2+}$ added to solution; however, there is still a relatively small amount (~23% of the total) of Zn$_3$-α-rhMT. Complete metallation was expected to occur when four Zn$^{2+}$ were added to apo-α-rhMT, but similar to the situation with the β-domain, analytical errors in concentrations and competitive formation of Zn(OH)$_2$ may impact the accuracy of the specified equivalents.

The relative abundance of each metallated α-rhMT species clearly demonstrates the stability of partially metallated species and thus provides evidence that metallation occurs in a non-cooperative manner for the C-terminal domain as well (Figure 3.4). At each equivalent of Zn$^{2+}$ added, with the exception of the beginning and end of the titration, α-rhMT also exists as a mixture of metallated states. At low equivalents of Zn$^{2+}$ a distribution of partially metallated species are observed, while at excess equivalents (> 4) of Zn$^{2+}$ the fully metallated α-rhMT is most abundant. Interestingly, there is a slight increase in the fraction of Zn$_2$-α-rhMT before Zn$_3$- and finally, Zn$_4$-α-rhMT form (Figure 3.4). The linear progression of metallation from apo-α-rhMT to Zn$_4$-α-rhMT provides further evidence that metallation occurs in a non-cooperative manner, where the declining affinity of Zn$^{2+}$ for α-rhMT is also directly related to the number of available metal-binding sites.

### 3.2.1 Metallation of rhMT 1a with Zn$^{2+}$

Similar to those for both the isolated β- and α-domains, mass spectra of the stepwise metallation of apo-βα-rhMT detail the formation of partially metallated species (from Zn$_1$- to Zn$_6$-βα-rhMT) at sub-stoichiometric equivalents (< 7) of Zn$^{2+}$, confirming the non-cooperative nature of the metallation reaction for the full human MT 1a (Figure 3.5). Only after 5.9 equivalents of Zn$^{2+}$ have been added to the solution does the holo-protein begin to form (Figure 3.5 E). It is not until excess Zn$^{2+}$ has been added that Zn$_7$-βα-rhMT becomes the most abundance species (Figure 3.5 F). Even at this point, there is still some (~12% of the total) Zn$_5$- and (~30%) Zn$_6$-βα-rhMT remaining. Similar to the situation with the individual domains, excess Zn$^{2+}$ is required, likely because of slight errors in the
Figure 3.4. Contour and bar graphs of the metallation of α-rhMT 1a as a function of Zn$^{2+}$ added. (A) A top-down view of metallation, showing the linear progression from apo-α-rhMT to Zn$_4$-α-rhMT as a function of Zn$^{2+}$ added to solution (100% abundance is shown in red and 0% abundance is shown in dark blue). (B) A side view of metallation, showing the relative abundance of each species as a function of Zn$^{2+}$ added. Colour code: apo-α-rhMT is red; Zn$_{1}$-α-rhMT is green; Zn$_{2}$-α-rhMT is dark blue; Zn$_{3}$-α-rhMT is light blue; Zn$_{4}$-α-rhMT is purple. Plots are based on the deconvoluted data in Figure 3.3. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
Figure 3.5. Mass spectra recorded during the titration of apo-βα-rhMT 1a with Zn\(^{2+}\). Spectra were recorded as aliquots of 7.3 mM ZnSO\(_4\) were titrated into a 29.2 µM solution of apo-βα-rhMT 1a (pH 9.1). (A-F) Charge states of βα-rhMT (left) and deconvoluted data (right) were recorded at Zn\(^{2+}\) molar equivalents of 0.9, 2.7, 3.7, 4.8, 5.9, and 8.4. Metallation in excess of Zn\(^{2+}\)-βα-rhMT is a result of non-specific adducts. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metalation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
estimation of the apo-protein concentration and the formation of trace amounts of competitive Zn(OH)$_2$ due to the high pH of the solution. Also similar to the Zn$^{2+}$ titration with the individual domains, the number of equivalents of Zn$^{2+}$ added dictates the dominant metallation state present in the full protein at each step in the titration. For example, after the addition of 3.7 equivalents of Zn$^{2+}$, the most abundant species was Zn$_4$-βα-rhMT (Figure 3.5 C). Likewise, when 5.9 equivalents of Zn$^{2+}$ were added, Zn$_6$-βα-rhMT was the most abundant species (Figure 3.5 E).

The relative abundance of each metallated βα-rhMT species demonstrates that the partially metallated species are stable and that metallation in human metallothionein 1a is non-cooperative (Figure 3.6). At each equivalent of Zn$^{2+}$, with the possible exception of the beginning and end of the titration, βα-rhMT exists as a mixture of at least three metallated states. At low equivalents of Zn$^{2+}$ only partially metallated species are observed, while at excess equivalents (> 7) of Zn$^{2+}$ only the fully metallated βα-rhMT is expected to be observed. Interestingly, one partially metallated species appears more prominent than the other intermediates; the Zn$_5$-βα-rhMT species occupies a greater fraction of the distribution than expected (Figure 3.5 D; also starred in Figure 3.6 B). The linear progression of metallation from apo-βα-rhMT to Zn$_7$-βα-rhMT demonstrates that metallation occurs in a non-cooperative manner, where the affinity of Zn$^{2+}$ for βα-rhMT is directly related to the number of available metal-binding sites.

3.2.1 A Stable Zn$_5$-βα-rhMT Intermediate

The increased stability of the intermediate Zn$_5$-βα-rhMT species may be of particular significance in the mettallation pathway of human metallothionein 1a. After 4.8 equivalents of Zn$^{2+}$ have been added to the solution of apo-βα-rhMT, the spectra are simplified with Zn$_5$-, and small amounts of Zn$_4$-βα-rhMT (~29%) and Zn$_6$-βα-rhMT (~14%) present (Figures 3.5 D and 3.6 B). This simplification of the distribution of species suggests that the binding constant ($K_F$) of Zn$_6$-βα-rhMT is significantly lower than that of Zn$_5$-βα-rhMT and that therefore, excess Zn$^{2+}$ must be added for MT to bind the sixth Zn$^{2+}$. 
Figure 3.6. Contour and bar graphs of the metallation of $\beta\alpha$-rhMT 1a as a function of $\text{Zn}^{2+}$ added. (A) A top-down view of metallation, showing the linear progression from apo-$\beta\alpha$-rhMT to $\text{Zn}_7-\beta\alpha$-rhMT as a function of $\text{Zn}^{2+}$ added (100% abundance shown in red and 0% abundance shown in dark blue). (B) A side view of metallation, showing the relative abundance of each species as a function of $\text{Zn}^{2+}$ added. Colour code: apo-$\beta\alpha$-rhMT is red; $\text{Zn}_1-\beta\alpha$-rhMT is green; $\text{Zn}_2-\beta\alpha$-rhMT is dark blue; $\text{Zn}_3-\beta\alpha$-rhMT is light blue; $\text{Zn}_4-\beta\alpha$-rhMT is light purple; $\text{Zn}_5-\beta\alpha$-rhMT is orange; $\text{Zn}_6-\beta\alpha$-rhMT is violet; $\text{Zn}_7-\beta\alpha$-rhMT is indigo. The star indicates the increased fraction of $\text{Zn}_5-\beta\alpha$-rhMT. Graphs are based upon the deconvoluted data in Figure 3.5. Reprinted with permission from Sutherland, D. E. K., Summers, K. L., and Stillman, M. J. (2012) Noncooperative metallation of metallothionein 1a and its isolated domains, Biochemistry 51: 6690-6700. © 2012 American Chemical Society.
The exact reason for this decrease in binding affinity is not clear from these data. I hypothesize that after the addition of five \( Zn^{2+} \), each of the \( Zn^{2+} \) are coordinated to four terminal cysteine residues and that binding of the sixth \( Zn^{2+} \) requires a structural rearrangement of these residues to form the first part of the expected two-domain structure. Previous studies have provided evidence to support the hypothesis that metals are initially coordinated to terminal cysteines before clustering takes place. However, these studies investigated only metals for which spectroscopic probes are available – not \( Zn^{2+} \). Several authors using a range of techniques, including \(^{113}Cd\)-NMR, Co-EPR and CD spectroscopies, to monitor metallation titrations have concluded that the initial metallation of MT must occur through exclusively terminal cysteine residues, after which cluster formation leads to bridging interactions (183, 201, 202).

From the results of a \( Cd^{2+} \) titration with rabbit liver MT 2 using NMR spectroscopy, it was proposed that the \( \beta\)-domain was metallated only after the complete saturation of the \( \alpha\)-domain (183). The authors fail to comment on a broad featureless peak at approximately 688 ppm that could be a \( Cd_5\)-MT species; the exclusively terminal coordination of \( Cd^{2+} \) to cysteine would cause significant fluctionality in the structure. Until their experiment is repeated using mass spectrometry to confirm the metal speciation as a function of metal loading, a domain specific, cooperative mechanism – at the high concentrations required for NMR spectroscopy – cannot be ruled out for \( Cd^{2+} \) binding to MT 2. At much lower concentrations (~10 µM), human MT 1a has been reported to bind \( Cd^{2+} \) non-cooperatively (120, 176).

An alternative hypothesis would be that a single \( Zn^{2+} \) binds in the \( \beta\)-domain and four \( Zn^{2+} \) bind cooperatively in the \( \alpha\)-domain. While reports on spectroscopic data have been interpreted in terms of a domain-specific, cooperative mechanism (resulting in the complete saturation of one domain before the other), thermodynamically, terminally bound thiols exhibit shorter bond lengths than bridged thiols and are, therefore, more energetically favourable. A complex involving twenty terminal S-\( Zn^{2+} \) bonds in a chain of five \( ZnCys_4 \) units would be thermodynamically more favourable than a \( Zn_4Cys_{11} \) cluster, a single \( ZnCys_4 \) unit, and five available cysteines. In addition, the cysteines
would be protected from oxidation in a structure with all-terminal coordination as opposed to the domain-specific mechanism.

3.2.2 Simulating Metal Binding Mechanisms

The Zn$^{2+}$ metallation properties of the isolated fragments and full protein were determined using sensitive, soft ESI MS methods (Figures 3.1 – 3.6). By analyzing the distribution of metallated states for both the rhMT and its isolated domains, it is evident that metallation occurs in a linear, step-wise fashion, which indicates that each Zn$^{2+}$ binds non-cooperatively (Figures 3.2, 3.4 and 3.6). Contrasting this mechanism is the ESI MS work of Gehrig et al. (92), which was interpreted to argue that Zn$^{2+}$ metallation of MT 2 proceeds in a cooperative manner.

The experimental mass spectra of the metallation of MT 1a with Zn$^{2+}$ (from Figure 3.1, 3.3, and 3.5) were plotted in terms of the 'life' of each Zn$_n$-MT species (where n=0-7) to further demonstrate that MT metallates via a non-cooperative mechanism (Figure 3.7 A, B, and C). This unique depiction of the individual metallated species highlights the distribution of species present at each point in the titration. Techniques that cannot discriminate between the various metallated species simply average the data from several species with different metallated states and, therefore, cannot provide the same level of information.

The stepwise metallation equations for $\alpha$-, $\beta$-, and $\beta\alpha$-rhMT 1a with Zn$^{2+}$ are shown below:

**Equation 1: Metallation of the $\beta$-domain peptide**

\[
apo - \beta - \text{rhMT} + Zn^{2+} \xrightarrow{K_{1\beta}} Zn_1 - \beta - \text{rhMT} \\
Zn_1 - \beta - \text{rhMT} + Zn^{2+} \xrightarrow{K_{2\beta}} Zn_2 - \beta - \text{rhMT} \\
Zn_2 - \beta - \text{rhMT} + Zn^{2+} \xrightarrow{K_{3\beta}} Zn_3 - \beta - \text{rhMT}
\]
Figure 3.7. Experimental and theoretical data showing Zn$^{2+}$ metallation through different binding mechanisms. (A, B, C) Speciation during metallation of the β- and α-domains, and the full MT protein with Zn$^{2+}$. (D, E, F) Simulation of ESI MS data using non-cooperativity rules (declining Ks). (G, H, I) Simulation using weakly cooperative binding rules (equal Ks). (J, K, L) Simulation using strongly cooperative binding rules (increasing Ks). Each line corresponds to a different metallation state: Zn$_0$(-----), Zn$_1$(--), Zn$_2$(••••), Zn$_3$(●●●●), Zn$_4$(----), Zn$_5$(-----), Zn$_6$(••••••), Zn$_7$(●●●●●●). Adapted from Biochemical and Biophysical Research Communications, 426, Sutherland, D. E. K., Summers, K. L., and Stillman, M. J., Modeling the Zn$^{2+}$ and Cd$^{2+}$ metallation mechanism in mammalian metallothionein 1a, 601-607. © 2012 with permission from Elsevier.
Equation 2: Metallation of the α-domain peptide

apo - α - rhMT + Zn^{2+} \xrightarrow{K_{1\alpha}} Zn_1 - α - rhMT
Zn_1 - α - rhMT + Zn^{2+} \xrightarrow{K_{2\alpha}} Zn_2 - α - rhMT
Zn_2 - α - rhMT + Zn^{2+} \xrightarrow{K_{3\alpha}} Zn_3 - α - rhMT
Zn_3 - α - rhMT + Zn^{2+} \xrightarrow{K_{4\alpha}} Zn_4 - α - rhMT

Equation 3: Metallation of the complete βα-rhMT 1a

apo - βα - rhMT + Zn^{2+} \xrightarrow{K_{1βα}} Zn_1 - βα - rhMT
Zn_1 - βα - rhMT + Zn^{2+} \xrightarrow{K_{2βα}} Zn_2 - βα - rhMT
Zn_2 - βα - rhMT + Zn^{2+} \xrightarrow{K_{3βα}} Zn_3 - βα - rhMT
Zn_3 - βα - rhMT + Zn^{2+} \xrightarrow{K_{4βα}} Zn_4 - βα - rhMT
Zn_4 - βα - rhMT + Zn^{2+} \xrightarrow{K_{5βα}} Zn_5 - βα - rhMT
Zn_5 - βα - rhMT + Zn^{2+} \xrightarrow{K_{6βα}} Zn_6 - βα - rhMT
Zn_6 - βα - rhMT + Zn^{2+} \xrightarrow{K_{7βα}} Zn_7 - βα - rhMT

This set of chemical equations represents the stepwise metallation of each protein where the binding affinities are represented by an equilibrium constant $K_n$ (where $n_\beta$ = 1 to 3; $n_\alpha$ = 1 to 4; $n_{βα}$ = 1 to 7). When Zn^{2+} is added under equilibrium conditions, the resulting products will depend on the relative $K_s$.

The experimental ESI MS data for Zn^{2+} metallasation of human MT 1a (Figures 3.1, 3.3, and 3.5) were compared to the predicted speciation from three mechanistic simulations (Figure 3.7 D–L). The theoretical metallasation curves for a non-cooperative system ($K_1$>$K_2$>$K_3$…>$K_n$) (Figure 3.7 D, E, F), a weakly cooperative system ($K_1$=$K_2$=$K_3$=…=$K_n$) (Figure 3.7 G, H, I), and a strongly cooperative system ($K_1$<$K_2$<$K_3$…<$K_n$) (Figure 3.7 J, K, L) were used to provide an unambiguous indication of the metallasation mechanism through a comparison of the simulation with the experimental ESI MS data. For MT, the binding affinities are expected to decrease as the binding sites fill, resulting in a distribution of metallasated species. The maximum abundance of each metallasated species is therefore expected to be approximately equal because the metallasation proceeds through a series of bimolecular reactions (shown above). In a weakly cooperative binding mechanism, the maximum intensity of all intermediate species, except Zn_1-MT, is
suppressed leading to the formation of fully metallated MT after the addition of only four equivalents of Zn\(^{2+}\) (Figure 3.7 G, H, I). This trend is further enhanced in the cooperative mechanism of metallation, where the intermediate species are even further suppressed and the fully metallated MT forms after the addition of only two equivalents of Zn\(^{2+}\) (Figure 3.7 J, K, L).

From these simulations (Figure 3.7 D–L), it is clear that if these proteins were metallated in a cooperative manner, holo-MT should appear in the mass spectra after the first equivalent of Zn\(^{2+}\) was added. The only abundant species would be the apo- and holo-protein; the partially metallated species would be present in low abundance or almost nonexistent at each step in a titration with Zn\(^{2+}\). A comparison between the experimental data and the modelled data provides strong evidence that metallation occurs via a non-cooperative mechanism (Figure 3.7).

The simulation can also be used to calculate the actual distribution of metallated species, similar to a mass spectrum, at each addition of Zn\(^{2+}\). The simulated deconvoluted mass spectra of βα-rhMT for two additions of Zn\(^{2+}\) (0.9 and 5.9 equivalents), based on the three mechanistic models, are easier to compare directly with experimental ESI MS data (Figure 3.8). The two points chosen provide a clear differentiation between the three mechanisms tested. In the non-cooperative mechanism, the dominant species is directly related to the number of equivalents of Zn\(^{2+}\) added to the solution, so that at 0.9 eq. of Zn\(^{2+}\) added, Zn\(_1\)-βα-rhMT is the most abundant; but at 5.9 eq. of Zn\(^{2+}\), Zn\(_6\)-βα-rhMT is the most abundant (Figure 3.8 B). The weakly cooperative mechanism demonstrates an initial metallation pattern similar to that of the non-cooperative mechanism; however, when a higher amount of Zn\(^{2+}\) is added, there is a much higher proportion of the fully metallated protein (Figure 3.8 C). As was expected, a cooperative mechanism favours either the apo-protein (prior to the addition of sufficient metal) or the holo-protein, with very low concentrations of partially metallated intermediates (Figure 3.8 D).

From the experimental mass spectra (Figures 3.1 – 3.6) and the simulations (Figures 3.7 and 3.8), it is clear that the mechanism of metallation of rhMT 1a with Zn\(^{2+}\) is non-cooperative. The model data demonstrate that there are significant differences in the
Figure 3.8. Experimental and simulated MS data for the metallation of apo-βα-rhMT 1a with Zn$^{2+}$. (A) Experimental MS data for βα-rhMT 1a with 0.9 and 5.9 equivalents of Zn$^{2+}$. Simulated MS data based upon non-cooperative (B) weakly cooperative (C) and cooperative (D) mechanisms. Adapted from Biochemical and Biophysical Research Communications, 426, Sutherland, D. E. K., Summers, K. L., and Stillman, M. J., Modeling the Zn$^{2+}$ and Cd$^{2+}$ metalation mechanism in mammalian metallothionein 1a, 601-607. © 2012 with permission from Elsevier.
metallation pattern of a cooperative and a non-cooperative mechanism and that ESI MS data are sensitive to these differences.

3.2.3 A Non-Cooperative Metallation Mechanism for rhMT 1a

Understanding the role of MT as a metallochaperone relies upon an understanding of its metallation properties, but the metallation properties of MT make the mechanistic study of the protein difficult. The coordination environment for all bound metal ions is similar. The affinity of the cysteinyl-thiolates for divalent metal ions (e.g., Zn$^{2+}$ and Cd$^{2+}$) is extremely high – too high to monitor kinetically. Spectroscopic probes cannot readily discriminate between the different metallated states of MT. As a result, the mechanism of metallation of mammalian MT has been intensively debated. Different reports present either a cooperative or non-cooperative mechanism of metallation in explanation of the data. Recent ESI MS data have provided significant new information about the early stages of metallation of metalloproteins (120, 168, 176, 203, 204), along with the discovery of the ‘supermetallated’ (metallation in excess of traditional levels) forms of MT (190, 191, 204).

In addition to the confusion regarding the mechanism, there has also been uncertainty as to the exact species formed following the addition of sub-saturating numbers of metal ions. Before metallation experiments were conducted using ESI MS, MT was often assumed to be homogenously metallated, meaning that the addition of one equivalent of M (the metal ion of interest) was interpreted as leading to the exclusive formation of M$_1$-MT, the addition of 2 equivalents of M resulted in precisely M$_2$-MT, and so on up to saturation. The metal distribution is represented by the ratio of the relative binding constants (K$_7$: K$_6$: K$_5$: K$_4$: K$_3$: K$_2$: K$_1$). Calculating the expected metal distribution in a stepwise titration and comparing it with the experimental data allowed the ratio of the relative binding affinities and consequently, the binding mechanism, to be determined. Only ESI MS data provide the exact distribution of metallated species at each point, allowing this comparison.

Initial studies of both the metallation and demetallation reactions of MT led to the hypothesis that metallation occurred in a cooperative fashion. There are three important
consequences of the cooperative mechanism. First, partially metallated forms would only ever represent a very low fraction (only fully metallated and fully metal-free would persist). Second, partially metallated forms would be unlikely to be available to play a major role in cellular chemistry and third, oxidation of the protein would lead to complete, cooperative demetallation of the protein.

Recent kinetic studies of As\(^{3+}\) metallation of hMT1 demonstrated conclusively that the affinity of MT for As\(^{3+}\) is directly dependent upon the number of available metal binding sites and that the specific rate constants for As\(^{3+}\) binding decline linearly as the number of bound As\(^{3+}\) increases, forming a chain of up to six cysteine-coordinated AsCys\(_3\) units \((111, 173, 200)\). If As\(^{3+}\) binding was cooperative, the specific rate constants would increase for each successive As\(^{3+}\) bound.

While the results presented above conclusively show that the mechanism of metallation of MT with Zn\(^{2+}\) is non-cooperative, the possibility that these results may be isoform specific cannot be ruled out. MT 1a metallates non-cooperatively with Cd\(^{2+}\), and As\(^{3+}\) \((120, 173, 176)\), and as shown herein, also with Zn\(^{2+}\), but MT 2a is still believed to metallate cooperatively with Zn\(^{2+}\), Cd\(^{2+}\) and Cu\(^{+}\) \((92, 183, 205)\). Recently, significant differences in the metallation chemistry of the MT isoforms have been noted. Both MT 1a and MT 3 are able to bind an eighth M\(^{2+}\) forming the ‘supermetallated’ M\(_8\)-βα-rhMT (where M\(^{2+}\) is Cd\(^{2+}\) for MT 1a and Zn\(^{2+}\) for MT 3; \((191, 203)\)), while MT 2 is thus far known to bind only seven M\(^{2+}\) ions forming M\(_7\)-βα-rhMT (where M\(^{2+}\) is Zn\(^{2+}\) or Cd\(^{2+}\)). These results provide further evidence to support a specific cellular function for each MT isoform.

Since the mechanism of metallation is non-cooperative for Zn\(^{2+}\), one of the most common metals bound by MT \textit{in vivo}, partially metallated species are presumably able to take part in cellular chemistry. In fact, instances in which partially metallated forms of the protein have been purified could represent the \textit{in vivo} forms of the protein \((206)\). In addition, MT may now be described as able to impede oxidative stress in a dynamic way. Progressive oxidation may lead to the sequential release of Zn\(^{2+}\) ions and eventually complete oxidation of the protein. The released Zn\(^{2+}\) may then bind to MTF-1 and lead to an
upregulation of genes for MT and other associated proteins, thus resisting oxidative stress (207). The balance between the metallated MT and the metal free MT was thought to mediate the redox chemistry of the cell (154, 208, 209); however, this view assumed a cooperative binding mechanism for metallation of metallothionein with zinc. It is possible that the average metal load of MT is what actually dictates the oxidative environment in a cell. DFT calculations of the reactivity of polynuclear zinc-thiolate sites have shown the mononuclear ZnCys4 group to be more nucleophilic than either of the two fully metallated Zn3Cys9- and Zn4Cys11-clusters (210). Given this information and the work presented here, Zn5-MT should be able to take part in cellular chemistry as a protective agent against oxidative stress even though it possesses no free thiol groups.

### 3.2.4 Summary

The results presented above demonstrate that Zn2+ metallation occurs in a non-cooperative manner, similar to Cd2+ and As3+ (111, 120, 173, 176), and currently in contrast to Cu+ (121). It is now clear that one of the two most significant metals in metallothionein cellular chemistry metallates in a non-cooperative manner. There is a prominent, stable Zn5-βα-rhMT species in the Zn2+ metallation pathway, which is hypothesized to be composed of terminally coordinated Zn2+ distributed in a series of ZnCys4 groups along the peptide chain. The transition from terminal to bridging Cys would reduce the affinity of the protein for Zn2+ and enhance the stability of Zn5-βα-rhMT. While domain specificity may exist, and Zn2+ may initially bind preferentially at either the N-terminus (β-domain) or the C-terminus (α-domain), domain formation likely occurs with the binding of the sixth Zn2+.

### 3.3 A Revised Structure of Holo-Metallothionein

The structure of metallated human metallothionein is thought to be that of a dumb-bell-like structure in which the two domains, β and α, are separated by an amino acid linker (as discussed in Section 1.4). This dumb-bell shape was first observed through the crystal structure described from X-ray diffraction data on rat liver Cd5Zn2MT 2 (69, 114). The structure of Zn7-MT has since been determined from 2D-1H NMR and is essentially identical to that of Cd7-MT (117). X-ray (69, 114), Cd-NMR (69, 115, 117), XAFS (137,
and modelling methods (211) all agree that Cd\textsubscript{7}-MT is a tight, two-domain structure dominated by Cd-thiolate cross-linking (94). The formation of the two metal-thiolate clusters is believed to occur in a way that allows different metal binding and stoichiometries in each domain. However, recent studies have provided evidence that this two-domain structure may be only one step in the complex metallation pathway of mammalian metallothionein (186, 191).

Metal binding in MT has been studied in a number of metallation, demetallation and metal replacement experiments conducted under equilibrium and kinetic conditions (95, 140). In particular, previous studies of the metal binding properties of human MT 2 with Zn\textsuperscript{2+} have shown a series of affinities that differ by four orders of magnitude (highest affinity K\textsubscript{F}=10\textsuperscript{11.8}, lowest affinity K\textsubscript{F}=10\textsuperscript{7.7}; (166)). This range of formation constants is thought to allow MT to act as a robust metallochaperone capable of accepting Zn\textsuperscript{2+} in cases of excess and donating Zn\textsuperscript{2+} in cases of deficiency. However, previous metallation experiments, such as copper luminescence, often provide only an averaged measure of metallation, not the exact speciation for all possible sites (118, 172). The discriminating capability of ESI MS provides information about all species present and their respective dynamic metallation states simultaneously. Because Zn\textsuperscript{2+} binding to MT is non-cooperative, the exact distribution at each step in the titration can be determined (186, 212).

Initially, the competition experiment between the two domains of human MT 1a described below was designed to help determine which domain filled first. The hypothesis that the \(\alpha\)-domain would metallate first was based on previous reports that provided evidence for the saturation of the \(\alpha\)-domain prior to the \(\beta\)-domain (183, 184), and that the \(\alpha\)-domain has one more Zn\textsuperscript{2+} binding site than the \(\beta\)-domain, although other studies have indicated that the \(\beta\)-domain may metallate preferentially (145).

The following competition experiment between the two domains and the full protein was designed to help determine the importance of the linker in the metallated structure. The full protein was initially hypothesized to metallate prior to saturation of the domains either because the two-domain structure arranged the Zn\textsuperscript{2+} binding sites in such a way
that would allow superior metal binding, or because of the advantage of its additional sites. However, in light of the non-cooperative nature of metallation and the increased stability of Zn\textsubscript{5}-\(\beta\alpha\)-rhMT (as determined in Section 3.1), it is not surprising that the data suggested an entirely different interpretation.

### 3.3.1 Competitive Metallation of \(\beta\)- and \(\alpha\)-rhMT \textit{1a} with Zn\textsuperscript{2+}

From the mass spectra recorded during a competition experiment in which incrementally added Zn\textsuperscript{2+} distributes between the \(\beta\)-domain (with three potential sites) and the \(\alpha\)-domain (with four potential sites), it was evident that the \(\alpha\)-domain did not bind all the Zn\textsuperscript{2+} ahead of the \(\beta\)-domain (Figure 3.9). In this experiment, both domains were present in equal concentration in the solution and thus the Zn\textsuperscript{2+} equivalents listed in Figure 3.9 are relative to the total protein concentration (\(\beta\)-domain + \(\alpha\)-domain = 7 binding sites). The charge state data are shown in Appendix A, Figure A.1.

The deconvoluted spectra show two distinct sets of peaks, which correspond to the different masses of the \(\beta\) and \(\alpha\) fragments (Figure 3.9). The data readily show the domain preference for each added Zn\textsuperscript{2+}; because there are seven binding constants in total, Zn\textsuperscript{2+} binding will be directed by the largest K\textsubscript{F}. The fractional distribution of Zn\textsuperscript{2+} between the domains changes drastically as Zn\textsuperscript{2+} loading increases.

The average metallation of the fragments (based on the data in Figure 3.9) gives a clearer picture of the Zn\textsuperscript{2+} distribution as increasing molar equivalents of Zn\textsuperscript{2+} were added (Figure 3.10). Between 0 and approximately 4 molar equivalents of Zn\textsuperscript{2+} added, the \(\beta\)-domain binds a greater fraction of the Zn\textsuperscript{2+}. After approximately 5 equivalents of Zn\textsuperscript{2+} were added, the \(\alpha\)-domain binds a greater fraction – likely because it still has two sites available. The \(\beta\)-domain clearly binds a greater fraction of the available Zn\textsuperscript{2+} up to Zn\textsubscript{2}-\(\beta\)-rhMT. The lines representing \(\beta\)-rhMT and \(\alpha\)-rhMT crossover when \(\beta\)-rhMT has bound two Zn\textsuperscript{2+}, probably because the \(\alpha\)-domain now has an advantage with two remaining sites compared with the single remaining site in \(\beta\)-rhMT (Figure 3.10) \(\textit{111}\).

Simulating the competition experiment allows the proposed mechanism to be tested. The experimental data for Zn\textsuperscript{2+} metallation of apo-\(\beta\)-rhMT and apo-\(\alpha\)-rhMT (split into two
Figure 3.9. Deconvoluted mass spectra recorded during the competitive titration of β- and α-rhMT with Zn$^{2+}$.

(A-F) Aliquots of 8.3 mM ZnSO$_4$ were added to the 29.2 µM apo-β- and apo-α-rhMT (pH 9.2) solution at molar equivalents of 0.4, 2.1, 3.5, 5.4, 7.6, and 8.0. The most intense species for both the β- and α-domain have been normalized to 100% relative abundance; Zn$^{2+}$ adducts are indicated by a star. The raw m/z data can be found in Appendix A, Figure A.1. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
Figure 3.10. The average metellation of β- and α-rhMT 1a as a function of Zn$^{2+}$ added. The β- (—) and α-domains (---) are present in equal concentrations. The plotted data were calculated from the spectra in Figure 3.9. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
to show the relative Zn$^{2+}$ binding for each domain) were compared with the simulation, which uses seven diminishing K_Fs to represent the seven total sites in the β- and α-domains (Figure 3.11). The K_F values were determined from the three-way competition experiment involving the two domains and the full protein (described in 3.2.2 and Figures 3.12–3.14). It is striking that the K_F model strongly mirrors the experimental data, with the β-domain initially binding in preference to the α-domain because the K_F values of the first two Zn$^{2+}$ binding sites in β-rhMT are higher.

3.3.2 Competitive Metallation of β-, α-, and βα-rhMT 1a with Zn$^{2+}$

When all three species (β-, α- and βα-rhMT) are mixed, they compete for added Zn$^{2+}$, but this time with fourteen binding sites that have different – but clearly very similar – K_F values. The experimental ESI MS data of this mixture containing the apo-β- and apo-α-domains, as well as the full apo-βα-protein, are even more surprising than the competition experiment between the two domains with Zn$^{2+}$ (Figure 3.12). The raw MS data are complicated because the charge states for the two domain peptides and the full protein at each metallated state overlap in the mass spectrum (Appendix A, Figure A.2). More information can be extracted from the deconvoluted mass spectra (Figure 3.12). By plotting the proportion of Zn$^{2+}$ bound by each protein (while in equilibrium with the other two species) up to full saturation of the proteins, the ‘life’ of the intermediate Zn$^{2+}$ species formed as Zn$^{2+}$ was added becomes more obvious (Figure 3.13). The average Zn$^{2+}$ bound by each protein can be readily determined by plotting the average metal loading of each species as a function of added Zn$^{2+}$ during competition (Figure 3.14).

The experimental data show that apo-βα-rhMT dominates Zn$^{2+}$ binding up to the point where 6.4 equivalents of Zn$^{2+}$ have been added (Figure 3.12 C). This is clearer from a comparison of the metallated species in solution (Figure 3.13 A, C, E); Zn_3 and Zn_4 have formed in the full βα protein, but only Zn_1 and Zn_2 have formed in the isolated domains after the addition of 6.4 equivalents of Zn$^{2+}$. If the binding constants were equal, the Zn$^{2+}$ would be distributed evenly between the three proteins (like in the weakly cooperative mechanism described in Subsection 3.15). The plots in Figure 3.13 (A, C, E)
Figure 3.11. Stepwise metallation of apo-β-rhMT and apo-α-rhMT in competition for added Zn\(^{2+}\). The metallation values for the β-domain (A) and the α-domain (C) were calculated from the MS data in Figure 3.9. Simulations of the metallation of the β-domain (B) and the α-domain (D) used \(\log_{10}(K_F)\) values of 12.7, 12.4, and 12.0 for the β-domain, and 12.6, 12.3, 11.8, and 11.5 for the α-domain. Each line corresponds to a different metallation state: Zn\(_0\) (●●●●), Zn\(_1\) (■■■■), Zn\(_2\) (▲▲▲▲), Zn\(_3\) (●●●●), and Zn\(_4\) (●●●●). Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, Biochemistry 52: 2461-2471. © 2013 American Chemical Society.
Figure 3.12. Deconvoluted mass spectra for the competitive titration of $\beta$-, $\alpha$-, and $\beta\alpha$-rhMT with Zn$^{2+}$. (A-F) Aliquots of 7.3 mM ZnSO$_4$ were added to a solution containing equimolar concentrations (29.9 $\mu$M each) of apo- $\beta$-, apo- $\alpha$-, and apo- $\beta\alpha$-rhMT (pH 7.8) at molar equivalents of 1.8, 4.6, 6.4, 9.3, 10.3 and 12.5. The most intense species for $\beta$-, $\alpha$- and $\beta\alpha$-rhMT have been normalized to 100% relative abundance. The titration stopped prior to 14 equivalents because of the noise from the high ZnSO$_4$ concentration. The raw m/z data are shown in Appendix A, Figure A.2. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
Figure 3.13. The stepwise metallation of apo-β-rhMT, apo-α-rhMT, and apo-βα-rhMT while in competition for added Zn$^{2+}$. The experimental data for the β- (A) and α-domains (C), and the full βα-rhMT (E) were calculated from the MS data shown in Figure 3.12. The simulation of the metallation of the isolated domains (B, D) and the full MT protein (F) used log$_{10}$($K_F$) values of 12.7, 12.4 and 12.0 for the β-domain, 12.6, 12.3, 11.8 and 11.5 for the α-domain, and 13.3, 13.1, 12.7, 12.4, 12.0, 11.3 and 10.8 for the full MT protein. Each line corresponds to a different metallation state: Zn$_0$ (---), Zn$_1$ (---), Zn$_2$ (---), Zn$_3$ (---), Zn$_4$ (---), Zn$_5$ (---), Zn$_6$ (---) and Zn$_7$ (---). Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, Biochemistry 52: 2461-2471. © 2013 American Chemical Society.
immediately indicate that the $K_F$s for the first four $\text{Zn}^{2+}$ in $\beta\alpha$-rhMT are greater than comparable $K_F$s in the two domains.

The simulation of the $\text{Zn}^{2+}$ distribution across the fourteen sites, when all three recombinant proteins are mixed and $\text{Zn}^{2+}$ is added stepwise, very closely models the experimental data (Figure 3.13 B, D, F). The $K_F$ values required in the model to explain the experimental distribution show that the first three sites in $\beta\alpha$-rhMT bind $\text{Zn}^{2+}$ preferentially because they have the highest $K_F$ values compared with those of the isolated domains. Because all sites are in equilibrium there appears to be a distribution between available sites, rather than a domain-specific mechanism.

The binding constants for the first three sites in $\beta\alpha$-rhMT were not initially expected to be greater than in the isolated domains because, at this stage in the metallation, the binding sites are composed of terminally bound $\text{Zn}^{2+}$ ($\text{ZnCys}_4$). However, the analysis of the $\text{As}^{3+}$ binding kinetics by Ngu et al. (111, 173, 174) predicted this effect. Because there are more available sites in $\beta\alpha$-rhMT than in the isolated domains, the $K_F$s of the first four sites of $\beta\alpha$-rhMT dominate metal binding. Analysis of the $\text{Zn}^{2+}$ data indicates the first three $\text{Zn}^{2+}$ bind to $\beta\alpha$-rhMT preferentially simply because of the effect of seven sites (compared to four in $\alpha$-rhMT and three in $\beta$-rhMT). However, the relatively simple model does not account for the greatly reduced $K_F$ for the sixth and seventh $\text{Zn}^{2+}$. An explanation of this effect is provided below.

### 3.3.3 Speciation during $\text{Zn}^{2+}$ Metallation of the Domains

The average metallation plots for the competition experiment in which the full MT protein and the isolated fragments compete for added $\text{Zn}^{2+}$ (Figure 3.14 A, B) were calculated from the experimental data (Figure 3.13 A, C, E). The average metallation plots are a complicated summary that may provide insight into the buffering and $\text{Zn}^{2+}$-donating properties of MT (Figure 3.14). There are three main features of the data shown in Figure 3.14 A. First, in the presence of $\beta\alpha$-rhMT, the $\beta$-domain binds $\text{Zn}^{2+}$ preferentially over the $\alpha$-domain – up to the binding of the second $\text{Zn}^{2+}$. The two traces for the isolated domains in competition with the full protein are almost identical to the
Figure 3.14. Experimental and simulated metallation of apo-β-, apo-α, and apo-βα-rhMT when competing for Zn$^{2+}$. (A) Average number of Zn$^{2+}$ bound in the β-domain (---), the α-domain (---------------), and the full MT protein (----) based on data in Figure 3.13. (B) Comparison of the average metallated states of the sum of the two domains (-----) with the full protein (---) as a function of added Zn$^{2+}$. (C) Simulation of the distribution of Zn$^{2+}$ between the three protein species using the log$_{10}$($K_F$) values of 12.7, 12.4 and 12.0 for the β-domain, 12.6, 12.3, 11.8 and 11.5 for the α-domain, and 13.3, 13.1, 12.7, 12.4, 12.0, 11.3 and 10.8 for the full MT protein (from Figure 3.13). Key features (1', 2', 3', 4', 5') in the experimental data (in A and B) are modelled in the simulation as well (1, 2, 3, 4, 5) (in C). Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
traces of the two domains without competition with the full protein (Figure 3.10), which clearly illustrates that the $K_F$ values remain constant. The simulations confirm that the $K_F$ values are consistent with the experimental data of two separate types of competition experiments. Second, the presence of $\beta\alpha$-rhMT reduces the overall $\text{Zn}^{2+}$ loading of the isolated domains compared with the $\text{Zn}^{2+}$ loading in the absence of $\beta\alpha$-rhMT (Figure 3.10). This is an important effect because it means that the isolated domains are binding proportionally less $\text{Zn}^{2+}$. This buffering property results from the equilibrium between all three species. Third, $\beta\alpha$-rhMT dominates $\text{Zn}^{2+}$ binding until the eighth $\text{Zn}^{2+}$ is added to the solution.

The average number of $\text{Zn}^{2+}$ sites filled in $\beta\alpha$-rhMT was compared with the sum of the $\text{Zn}^{2+}$ sites filled in the isolated $\beta$- and $\alpha$-domains to provide a clearer indication of the difference between two linked domains and two separate domains (Figure 3.14 B). If the $K_F$s were the same for each $\text{Zn}^{2+}$ site (i.e., the $K_F$s for first site in $\beta\alpha$, $\beta$, and $\alpha$ were identical), then the two lines should be coincident. Clearly, $\beta\alpha$-rhMT binds more $\text{Zn}^{2+}$ into its sites compared with the same number of sites in the domains (i.e., $\beta$- plus $\alpha$-rhMT), but only until the eighth $\text{Zn}^{2+}$ is added. Afterward, the isolated domains preferentially bind more $\text{Zn}^{2+}$ even though the same number of sites is available in the full protein.

A simulation of the stepwise distribution of $\text{Zn}^{2+}$ was performed based on the same $K_F$s as were used previously and it matches the experimental data in Figure 3.14 (A, B) almost exactly (Figure 3.14 C). The five key features (labeled 1’-5’) on the lines in Figure 3.14 (A, B) are reproduced exactly in the simulation (labeled 1-5; Figure 3.14 C), confirming the reliability of the $K_F$ values used in the simulation. This provides further support for the model, which considers diminishing $K_F$ values for all sites as well as the advantage of the seven sites in $\beta\alpha$-rhMT over the 4 + 3 sites in the isolated domains.

3.3.4 Cluster Formation: A Greater Reduction in $K_F$ for the Sixth $\text{Zn}^{2+}$

The isolated domains begin to bind more $\text{Zn}^{2+}$ just as the sixth $\text{Zn}^{2+}$ binds to $\beta\alpha$-rhMT. Structurally, this is where $\beta\alpha$-rhMT must use bridging cysteiny1 sulfurs to form the
$\text{Zn}_6\text{Cys}_{20}$ structure from the string of five isolated, terminally coordinated $\text{ZnCys}_4$ sites that form the $\text{Zn}_3\text{Cys}_{20}$ structure. The simulation shows that the $K_F$s for the sixth and seventh $\text{Zn}^{2+}$ sites in $\beta\alpha$-rhMT are lower than the $K_F$s of the fourth $\alpha$-rhMT site and the third $\beta$-rhMT site and, therefore, the isolated domains preferentially bind the incoming $\text{Zn}^{2+}$ (Figure 3.13). The first three $\text{Zn}^{2+}$ binding sites in $\beta\alpha$-rhMT exhibit higher $K_F$ values than any sites in the isolated domains. Following the advantage of the seven available sites in $\beta\alpha$-rhMT compared to the three in $\beta$-rhMT and the four in $\alpha$-rhMT, the $K_F$ values of all three proteins are balanced and approximately equal once the fifth $\text{Zn}^{2+}$ binds to $\beta\alpha$-rhMT (Figures 3.13 B, D, F and Figure 3.14 C). After this point, the isolated domains metallate at the expense of the full protein – most likely because cluster formation in the isolated domains is not as disruptive. The drop in binding affinity for the sixth and seventh $\text{Zn}^{2+}$ sites in $\beta\alpha$-rhMT is based on the properties of cysteine ligands. The onset of bridging cysteiny1 thiolates in $\beta\alpha$-rhMT leads to a significant decrease in $K_F$ after five $\text{Zn}^{2+}$ have bound terminally to all twenty cysteines. The data suggest that it is the sixth $\text{Zn}^{2+}$, with a lower affinity for $\beta\alpha$-rhMT compared to the isolated domains, that causes bridging to occur and forces the two domain structure to form. The model suggests massive rearrangements are necessary to change the structure from that of five $\text{ZnCys}_4$ units to the clustered, domain-separated $\text{Zn}_7\text{Cys}_{20}$ structure (Subsection 3.2.7). The seventh $\text{Zn}^{2+}$ completes the two-domain structure leading to the $\text{Zn}_4\text{Cys}_{11}$ $\alpha$-domain and the $\text{Zn}_3\text{Cys}_9$ $\beta$-domain.

In summary,

$$\text{apo-}\text{Cys}_{20}-\beta\alpha\text{-rhMT} + 5 \text{Zn}^{2+} \rightarrow (\text{ZnCys}_4)_5-\beta\alpha\text{-rhMT}$$

$$(\text{ZnCys}_4)_5-\beta\alpha\text{-rhMT} + 2 \text{Zn}^{2+} \rightarrow (\text{Zn}_4\text{Cys}_{11} + \text{Zn}_3\text{Cys}_9)-\beta\alpha\text{-rhMT}$$

### 3.3.5 Clues from $\text{As}^{3+}$ Binding Kinetics Studies

Previous metal binding studies of rhMT 1a with $\text{As}^{3+}$ demonstrated that the metal-binding affinity of MT is directly dependent on the number of available binding sites. In fact, the $K_F$s essentially decreased from the first $\text{As}^{3+}$ bound to the sixth bound (recall that $\text{As}^{3+}$
binds to MT using terminal cysteines only) (111, 173, 174, 200). Interestingly, a similar trend is observed here with Zn$^{2+}$ (Figure 3.13).

From the results above, it is apparent that the hypothesis by Ngu et al. (111, 173, 174, 200), in which $K_F$s decrease from the first to the last metal bound, holds true up to the fourth and fifth Zn$^{2+}$ bound. The considerable difference between the two metal-ion species is that Zn$_7$-rhMT requires bridging cysteines, while bridging cannot occur in As$^{3+}$ metallation, leading to As$_6$-MT as the saturated species. Interestingly, the key species in Zn$^{2+}$ metallation is the terminally coordinated Zn$_5$-βα-rhMT, where the $K_F$ for the conversion of Zn$_5$-βα-rhMT to Zn$_6$-βα-rhMT is sufficiently low to allow near quantitative formation of the Zn$_5$ species.

Details from the As$^{3+}$ studies, along with the new information obtained from the ESI MS data described above, are crucial in placing Zn-MT between Zn$^{2+}$ sensors that must be present in mammalian cells (e.g., sensors similar to the bacterial ZiaR (85) and ZUR (213)) (Subsection 1.3.3). By considering the results presented here in the context of the cellular Zn$^{2+}$ sequestration equilibrium, MT may play a key role in Zn$^{2+}$ storage, trafficking, and donation. Three main characteristics demonstrate the flexibility of MT and may provide insight to the role of MT in Zn$^{2+}$ homeostasis. First, it is evident that the $K_F$ values are proportional to the number of available binding sites. Second, there does not appear to be a domain preference for Zn$^{2+}$ – at least until terminal thiolates bridge to bind the sixth and seventh Zn$^{2+}$. Third, bridging interactions likely lead to the significantly decreased $K_F$ values observed. Taking these three points into consideration, donation of the weakly bound sixth and seventh Zn$^{2+}$ to apo-enzymes would not expose any of the twenty cysteines to oxidation and would allow subsequent acquisition and donation. However, the redox situation will be more complicated in vivo, most likely involving the reducing effects of glutathione. In effect, the sixth and seventh Zn$^{2+}$ may act as the buffer-active Zn$^{2+}$ with lower $K_F$s than the first five structural Zn$^{2+}$.

### 3.3.6 Clues from Previously Published Co$^{2+}$ EPR Data

Cobalt, with its rich spectroscopic properties and characteristic tetrahedral coordination with thiols, has been extensively used to mimic Zn$^{2+}$ binding to MT. Cobalt EPR is
unique because the intensity is directly related to the number of Co\(^{2+}\) bound to MT and the number of non-clustered Co\(^{2+}\) centers. In work by Vasak and Kagi (201), metallation of MT with Co\(^{2+}\) was monitored using EPR spectroscopy. Analysis of the data, when plotted as a function of added Co\(^{2+}\), showed an increase in the number of free spins until four molar equivalents had been added. After this point, antiferromagnetic coupling led to a decrease in the signal intensity, which approached zero after the addition of seven equivalents of Co\(^{2+}\). These data were interpreted as cluster formation taking place after four metal ions had been added.

The EPR experiment was simulated using the Zn\(^{2+}\) data for \(\beta\alpha\)-rhMT described above (Subsection 3.2.2), which shows the distribution of metals in non-clustered sites (up to five Zn\(^{2+}\) bound) and clustered sites (six and seven Zn\(^{2+}\) bound). A fictitious spin was assigned to the non-bridging Zn\(^{2+}\) to mimic Co\(^{2+}\) spins and the spins were cancelled when clustering occurred (Figure 3.15). The following rules were used to simulate the predicted, fictitious magnetic moment: Zn\(_1\)- through Zn\(_5\)-rhMT form without any bridging interactions and, consequently, increase the EPR signal; the formation of Zn\(_6\)- and Zn\(_7\)-rhMT require four and eight bridging interactions, respectively and, consequently, decrease the signal due to antiferromagnetic coupling. The simulated EPR data closely resemble the original Co\(^{2+}\) data with an identical maximum at four metals added (the average number of spins are shown rather than the calculated g factor) (Figure 3.15). From the model with five terminally bound M\(^{2+}\), five M\(^{2+}\) would be predicted to result in maximal spin intensity; however, the non-cooperative nature of metallation requires that some M\(_6\)-rhMT must also exist at this metal concentration. In the Co\(^{2+}\) titration, this species would greatly decrease the signal intensity. The close similarity between the modelled data (which are based upon ESI MS speciation data) and the original Co\(^{2+}\) data, provides support for the proposal that clustering does not occur until the sixth metal ion is added and that Zn\(_5\)-rhMT is a single domain species that does not involve bridging cysteines.
Figure 3.15. Simulated EPR data based on metellation of apo-βα-rhMT with Zn$^{2+}$. The simulated average number of spins was calculated assuming Zn$^{2+}$ has the same spin as Co$^{2+}$. The exact metellation state was obtained from analysis of the data in Figure 3.12. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
3.3.7 Molecular Modelling of a Potential Metallation Pathway

A series of molecular dynamics calculations were used to illustrate the structural changes that occur during the progressive Zn$^{2+}$ metallation of apo-βα-rhMT to the intermediate Zn$_5$-βα-rhMT, and finally to Zn$_7$-βα-rhMT (Figure 3.16). The minimized apo-βα-rhMT structure resembles that reported by Rigby et al. (126, 127, 129), showing a globular fold with the free cysteinyl thiols aligned on the surface (Figure 3.16 A). Tetrahedral Zn$^{2+}$ coordination by terminal cysteines was arranged for the first five Zn$^{2+}$, forming a single Zn$_5$Cys$_{20}$ binding domain. It is important to note that the Zn$_5$- and Zn$_6$-βα-rhMT species presented are of a qualitative nature (Figure 3.16 B, C). Without definitive structural information, alternative connectivities may be possible. Despite the lack of cluster formation in Zn$_5$-βα-rhMT, the overall three-dimensional structure of the protein resembles that of fully metallated Zn$_7$-βα-rhMT. Even when there are no bridging cysteines present, the Zn$_5$-βα-rhMT model shows two Zn$^{2+}$ in the β-domain cysteines (within residues 1 to 36) and three Zn$^{2+}$ in the α-domain cysteines (within residues 37 to 74), which are spatially close to one another (Figure 3.16 B). This likely facilitates cluster formation upon further metallation to the traditional two-domain Zn$_7$-βα-rhMT (Figure 3.16 D).

3.3.8 Summary

The data presented in this section are believed to show that, with the onset of cluster formation (as defined by the use of bridging rather than terminal cysteines), there is a significant decrease in the Zn$^{2+}$ binding affinity of MT. This is highlighted in the simulation with the decrease in K$_F$. From the simulation of the MS data, the decreasing K$_Fs$ of a non-cooperative metallation mechanism appear to lead to formation of a stable Zn$_5$-βα-rhMT species, without bridging interactions. This is likely a key member of the metallation pathway from apo-rhMT to the saturated Zn$_7$-rhMT.

The sixth and seventh Zn$^{2+}$, therefore likely act as the buffer-active Zn$^{2+}$ with lower K$_Fs$ than the first five structural Zn$^{2+}$, allowing MT to donate and acquire Zn$^{2+}$ in vivo without oxidizing the cysteines. In the cellular environment, this positions Zn-MT as the main
Figure 3.16. Molecular models of possible structures formed in the metallation pathway from apo-rhMT to Zn\(_7\)-rhMT. Structural models are shown on the left and the amino acid sequences of \(\beta\alpha\)-rhMT showing the Zn\(^{2+}\) connectivities are shown on the right. The backbone is depicted as a ribbon, with green spheres for Zn\(^{2+}\) and yellow spheres for the sulfur atoms. The N-terminal \(\beta\)-domain is located on the left and the C-terminal \(\alpha\) domain is located to the right. (A) Metal-free, apo-\(\beta\alpha\)-rhMT (structure first reported by Rigby et al. (2004) (126)). (B) Single-domain Zn\(_5\)-\(\beta\alpha\)-rhMT (structure created by assigning each set of four cysteines to one Zn\(^{2+}\)). (C) A possible structure for Zn\(_6\)-\(\beta\alpha\)-rhMT using cysteines known to bridge in the saturated two-domain structure. (D) Formation of Zn\(_7\)-\(\beta\alpha\)-rhMT with rearrangement of bound metals triggered by the addition of the seventh Zn\(^{2+}\). Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
Zn$^{2+}$ storage protein, buffering intracellular Zn$^{2+}$ levels between the detection limits of Zn$^{2+}$ sensors (such as Zia (85) and ZUR (213) in microorganisms) and within the Zn$^{2+}$ sequestration equilibrium.

In a recent study by Sutherland et al. (191) on the ‘supermetallation’ of MT 1a, the addition of an eighth Cd$^{2+}$ caused the two domains to coalesce into one ‘super-domain’. When considered with the results presented here, a fundamentally altered view of the metallation properties of MT emerges. A new structural characterization of MT where MT exists as a flexible, single domain protein during initial metallation ($< 5$ equivalents) and metallation in excess of traditional levels ($> 7$ equivalents) seems more plausible than a rigid structure where the two domains are maintained regardless of metal depletion.
4 Summary and Conclusions

New studies – particularly using ESI MS – are providing evidence for an altered binding mechanism for metallothioneins. Before the application of ESI MS to the study of MT metallation, many of the intermediate species were not observed, leading researchers to believe that the protein bound metals cooperatively. Similarly, without ESI MS the enhanced stability of the Zn$_5$-MT species was not observed. Recently, with much wider use of ESI MS, MT has been proposed to exhibit even more flexibility in metal binding. In fact, MT 1a is believed to bind metals in a step-wise manner that leads to the stable M$_5$-MT, then saturation leads to the formation of the well-described M$_7$-MT, and finally, excess leads to the formation of a single-domain, ‘supermetallated’ M$_8$-MT (where M is a divalent metal, specifically Zn$^{2+}$ or Cd$^{2+}$) (191). With this new information, the in vivo roles of MT must be reassessed; MT is now hypothesized to be a much more flexible protein with roles in metal buffering and donation, as well as in reduction of reactive oxygen species. MT may be able to fulfill all these roles simultaneously, with partially metallated and partially oxidized intermediate species existing to buffer intracellular metal concentrations and oxidative stress.

4.2 ESI MS in Metal Binding and Structural Studies

Electrospray ionization mass spectrometry has provided key information about the mechanism of mettallation of MT – specifically the metal ion speciation as a function of metals added. Without secondary structure, traditional spectroscopic probes do not provide significant information about MT – especially not the apo-protein (100-102). In addition, the essential Zn$^{2+}$ stored by mammalian MT, is spectroscopically invisible (165). Cadmium has been used as a structural probe for Zn$^{2+}$ mettallation in many of the techniques used to study metalloproteins because it is isostructural to Zn$^{2+}$, because mammalian Cd-MT and Zn-MT structures are almost identical, and because it exhibits spectral properties amenable to standard spectroscopic analyses (e.g., UV absorption, CD and NMR spectroscopies) (116, 117, 140).

ESI MS has allowed the direct analysis of Zn-MT (168-170). ESI MS permits the identification of the number and type of metal ions bound by analyzing any changes to
the protein mass and charge states. Conversely, spectroscopic techniques often only provide an average of the total metals bound. Prior to mass spectrometric analysis, solutions of MT were thought to be homogeneous (i.e., if 1 equivalent of Cd\textsuperscript{2+} was added, the solution would contain 100% Cd\textsubscript{1}-MT); however, it is now evident that solutions of MT are often a distribution of metallated species (120).

Without ESI MS, the mechanism of metallation of metallothionein could not be understood. For example, the observation of a number of intermediate species provided evidence for a non-cooperative binding mechanism. Because ESI MS can monitor multiple species simultaneously (provided they have different masses), the competition studies that ultimately resulted in stronger evidence for an important Zn\textsubscript{5}-MT species and relative binding constants for each Zn\textsuperscript{2+} binding site in metallothionein were devised and successfully carried out.

4.3 Non-Cooperative Zn\textsuperscript{2+} Binding to MT 1a

Many proteins do not exhibit any form of cooperativity in binding of their substrates. In hindsight, the hypothesis that a relatively simple protein such as metallothionein would bind metals cooperatively seems somewhat unlikely. Without any secondary structure in the form of \(\alpha\)-helices or \(\beta\)-sheets, MT is quite flexible. MT demonstrates metal-dependent protein folding, where metal binding dictates the tertiary structure of the protein; therefore, upon saturation with metals, MT can be described as having a rigid tertiary structure in the form of two distinct metal-thiolate clustered binding domains. It is difficult to believe that binding of one metal would then increase the affinity for additional metal ions, especially when terminally bound metals are energetically more favourable than metal binding sites that require bridging thiolates.

The results presented in this study demonstrate that the metallation of MT with Zn\textsuperscript{2+} occurs in a non-cooperative fashion and that partially metallated species may be critical in the intracellular balance of metal ions. Metallothionein does not simply exist as a mixture of apo- and holo-protein as predicted by a cooperative metallation mechanism. The intermediate species between the apo- and holo-MT proteins may be key to the metal buffering capability of MT.
4.4 The Role of Metallothionein in Zn$^{2+}$ Homeostasis

Metallothionein is often implicated in metal-ion homeostasis and toxic metal detoxification. MT has been shown to act as a metallochaperone, capable of transporting essential metal ions, such as Cu$^{+}$ and Zn$^{2+}$, in a controlled manner. In this role, MT must be able to deliver metal ions to apo-enzymes. To investigate this characteristic, metal exchange experiments have been conducted in which Zn$^{2+}$ from Zn$_{7}$-$\beta$-MT has been transferred to Zn$^{2+}$-dependent enzymes. For example, m-aconitase (155), carbonic anhydrase (156, 157) and the Gal4 transcription factor (158) can receive Zn$^{2+}$ from MT. Similarly, MT has also been shown to accept Zn$^{2+}$ from the zinc finger-containing transcription factor Sp1 (159).

Electrospray ionization mass spectral studies have previously highlighted the stability of partially metallated MT during the transfer of Zn$^{2+}$ from Zn-MT to metal ion chelators or to an apo-enzyme (160-162). However, the results described in this thesis provide evidence for a significantly different way in which MT could buffer intracellular zinc concentrations.

4.4.1 The Importance of Zn$_{5}$-rhMT 1a

The structures of partially metallated MT intermediates, as well as the structure of saturated Zn$_{7}$-MT, are critical to understanding the donation of Zn$^{2+}$ to apo-enzymes. Donation of Zn$^{2+}$ from the saturated protein, with collapse of the clustered binding site structure (as proposed by a cooperative binding mechanism), would lead to subsequent exposure of the cysteinyl thiols to the cellular environment and oxidation would rapidly result (154, 155, 157, 158). While MT has been shown to accommodate Zn$^{2+}$ into two metal-cysteine clusters when saturated, experiments detailed herein show that Zn$^{2+}$ binds in two steps. Electrospray ionization mass spectrometric analysis of Zn$^{2+}$ metallation and simulation calculations provided the relative binding affinities, and MT was proposed to contain five strong binding sites and two weak binding sites. The first five Zn$^{2+}$ are now believed to bind sequentially, then the clusters form with binding of the sixth and seventh Zn$^{2+}$. 
Initial metallation of MT is no longer thought to occur in a domain specific manner, but rather the first five $\text{Zn}^{2+}$ are believed to bind terminally to the twenty thiolates in a sequential fashion. Subsequent metallation to form $\text{Zn}_6$- and $\text{Zn}_7$-MT requires a complete structural rearrangement, loss of the single domain structure, and formation of the clustered domains. Clustering takes place through bridging interactions of the thiolates, which causes the associated reduction in $K_F$ for the last two sites. Donated $\text{Zn}^{2+}$ is thought to come from the clustered $\text{Zn}_7$-MT and, following donation, the terminally bound $\text{Zn}^{2+}$ structure re-forms, protecting the thiols from exposure to oxidation (Figure 4.1). This terminally coordinated $\text{Zn}_5$-MT species can then accept $\text{Zn}^{2+}$ from metallochaperones.

On a slightly different note, the metal-binding-induced structure of MT was believed to require translation of the entire polypeptide chain before metallation and folding could occur, which would result in the exposure of the twenty, highly reactive thiols to the intracellular environment (126). In light of the evidence to support a $\text{Zn}_5$ intermediate species, I propose an alternative hypothesis. Specifically, $\text{Zn}^{2+}$ may bind to the MT peptide as each set of four cysteines emerges during translation, resulting in the $\text{Zn}_5$-MT upon completion of protein translation. After this point, an additional two $\text{Zn}^{2+}$ may bind to induce formation of the well-described, two-domain $\text{Zn}_7$-MT structure. When recent studies on ‘supermetallated’ Cd$_8$-MT are also considered, it would appear that the two-domain M$_7$-MT species so often described in structural studies is a unique point in the metallation pathway of MT. MT may exist as a single domain protein with up to five M$^{2+}$ bound and with more than seven M$^{2+}$ bound.

### 4.5 The Role of Metallothionein in Oxidative Stress

Metallothionein is often cited as a protective agent against oxidative stress, where the balance between apo- and holo-MT controls the redox chemistry of the cell (154, 208, 209). Considering the non-cooperative nature of the metallation of MT with zinc, the average metal load of MT may somewhat dictate the oxidative environment of the cell. Even though $\text{Zn}_5$-rhMT possesses no free thiol groups, $\text{Zn}_5$-MT should be able to take part in cellular chemistry as a protective agent against oxidative stress.
Figure 4.1. Schematic showing the cyclic acquisition and donation of Zn$^{2+}$ by metallothionein. Zn$^{2+}$ are represented as green spheres and cysteiny1 sulfurs are represented as yellow spheres. Zn$_5$-rhMT may buffer slight increases in intracellular Zn$^{2+}$ concentrations by accommodating an additional two Zn$^{2+}$. Similarly under slightly limiting intracellular Zn$^{2+}$ concentrations, Zn$_7$-rhMT may donate two weakly bound Zn$^{2+}$ to Zn$^{2+}$ dependent apo-enzymes. The ‘Zn-Chaperone’ and the ‘Zn-Enzyme’ are a ZIP protein (PDB structure 2AJ0) and carbonic anhydrase (PDB structure 3U3A), respectively (71, 214). Adapted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, Biochemistry 52: 2461-2471. © 2013 American Chemical Society.
Equilibria between apo-MT, oxidized MT and holo-MT have been suggested to affect the stress response of organisms (208); however, metallation of MT was assumed to occur through a cooperative mechanism. A recent report on the isolation of partially oxidized MT from mice under oxidative stress provides further support for a role of partially metallated MT as a protective agent against oxidative stress (206). MT is able to impact the pathogenesis of diseases such as hyperglycemia, ischemic cardiomyopathy, type 2 diabetes, and cardiovascular complications through Zn\(^{2+}\) buffering and antioxidant dysfunction, both of which rely on the metallation status of MT in vivo (215-217). The knowledge that MT metallates non-cooperatively will aid in determining the function of MT in these disorders. From the evidence reported here on the metallation of MT, it is possible that MT is able to combat oxidative stress in a dynamic way, where progressive oxidation of MT leads to the sequential release of Zn\(^{2+}\) ions and eventually complete oxidation. This released Zn\(^{2+}\) can then bind to MTF-1 (207), Zn-MTF-1 then leads to an upregulation of MT (and other Zn\(^{2+}\) binding proteins) and resistance to the oxidative stress.

4.6 Future Directions

The novel ESI MS studies described in this thesis have opened many avenues for further study of MT and its roles in the homeostasis of essential metals, detoxification of toxic metals, and in protection against oxidative stress. Future studies should attempt to provide concrete evidence for the potential structures detailed in Figure 4.1. Crystal structures of Zn\(_5\)-MT, Zn\(_6\)-MT, and Zn\(_7\)-MT would greatly impact the understanding of the structure, and therefore, function of metallothioneins in the homeostasis of zinc. In addition, because terminal metal-thiolate bonds are thought to be shorter than bridging bonds, a difference in M-S bond length may be observed in XAFS studies of the terminally coordinated sites in Zn\(_5\)-MT compared with the clustered binding sites in Zn\(_7\)-MT. With the understanding that obtaining a crystal structure is no small task, there may be other ways to investigate the stable Zn\(_5\)-MT and its potential role in buffering zinc concentrations within a cell. For example, the available cysteines can be counted using a covalent cysteine modifier, parabenzoquinone, which has been used previously to investigate the structure of apo-MT and the accessibility of partially metallated MT (130,
Modification of a cysteine with benzoquinone results in a mass shift of 108.09 Da from the mass of the protein and is thus easily identified in a deconvoluted mass spectrum. If the Zn$^{2+}$ in Zn$_5$-MT are indeed coordinated only by terminal cysteines, there should be no sulfurs available to bind the benzoquinone. In fact, the use of benzoquinone as a modifier of available cysteines may aid in the elucidation of the various intermediate structures in the metallation pathway of MT.

Further investigation into the significance of the Zn$_5$-rhMT intermediate species, through interactions of Zn$_7$-rhMT with Zn$^{2+}$-dependent apo-enzymes, is also required. If formation of a Zn$_5$-species permits donation to apo-enzymes with binding affinities on the order of $10^{11}$ M$^{-1}$, Zn$_7$-MT should readily donate two Zn$^{2+}$. In addition, a sample of 100% Zn$_5$-MT should readily accept two Zn$^{2+}$ from Zn$^{2+}$ transport proteins with lower K$_F$s than the sixth and seventh binding sites in $\beta\alpha$-rhMT (on the order of $10^{10}$ M$^{-1}$).

Now that the metallation mechanism for Zn$^{2+}$ has been well characterized, with non-cooperative binding and a stable Zn$_5$-rhMT intermediate, similar experiments are required to decipher the binding mechanism of the other essential metal often bound by MT in vivo, Cu$^+$. Copper coordination is different from that of Zn$^{2+}$ (digonal or trigonal vs. tetrahedral) and therefore, the metallation pathway is not expected to be identical. It would be of interest to investigate the existence of an intermediate Cu-MT species that coordinates the Cu$^+$ using only terminal cysteines. Investigation of the Cu$^+$ metallation of MT would be slightly more difficult than that of Zn$^{2+}$ because not only will the twenty cysteinyl sulfurs oxidize, but so will the Cu$^+$ if exposed to oxygen.

The two-domain structure of many metallothioneins is thought to allow the protein to function simultaneously in both Cu$^+$ and Zn$^{2+}$ homeostasis. Future metallation studies in which metallothionein is exposed to increasing amounts of both Cu$^+$ and Zn$^{2+}$ are needed to determine if the metallation pathway for metallothionein is different in the presence of both metals. In particular, a competition experiment in which the domain peptides compete for Cu$^+$ and Zn$^{2+}$ may give some indication of any domain preference for these metals. However, such an experiment may not be easily monitored by ESI MS because the masses of copper and zinc are quite close (63.55 and 65.38 g/mol, respectively).
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References


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Appendix: Charge State Spectra

The following Figures, A1 and A2, are the charge state spectra that complement the deconvoluted spectra (Figures 3.9 and 3.12, respectively). Figure A1 corresponds to the $m/z$ spectra recorded for the competitive titration of Zn$^{2+}$ between the two isolated domain peptides. Figure A2 corresponds to the $m/z$ spectra recorded for the competitive titration of Zn$^{2+}$ with the two domain peptides and full MT protein.
Figure A.1. Charge state spectra recorded during the competitive titration of β- and α-rhMT with Zn$^{2+}$. Spectral changes were recorded as aliquots of 8.3 mM ZnSO$_4$ were added to the solution of 29.2 µM apo-β- and apo-α-rhMT (pH 9.2) on ice. Mass spectra were recorded at Zn$^{2+}$ molar equivalents of 0.4, 2.1, 3.5, 5.4, 7.6, and 8.0. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
Figure A.2. Charge state spectra recorded during the competitive titration of β-, α-, and βα-rhMT with Zn$^{2+}$. Mass spectra were recorded as aliquots of 7.3 mM ZnSO$_4$ were added to a solution containing equimolar concentrations (29.9 µM) of apo-β-, apo-α-, and apo-βα-rhMT (pH 7.8) at molar equivalents of 1.8, 4.6, 6.4, 9.3, 10.3 and 12.5. Reprinted with permission from Summers, K. L., Sutherland, D. E. K., and Stillman, M. J. (2013) Single domain metallothioneins: Evidence for the onset of clustered metal binding domains, *Biochemistry* 52: 2461-2471. © 2013 American Chemical Society.
Curriculum Vitae

Name: Kelly Lynn Summers

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2012-2013 M.Sc. Biology

The University of Western Ontario
London, Ontario, Canada
2007-2012 B.Sc. (Honors) Biology

Honours and Awards:
NSERC Canadian Graduate Scholarship for 2013-2014
Invited to speak at CanBIC-4 in Parry Sound, Canada (2013)
Travel scholarship to attend 5th International IMBG Meeting in Autrans, France (2012)
Western Science Graduate Entrance Scholarship (2012)
Prize awarded for honors research presentation (2012)

Related Work Experience:
Graduate Teaching Assistant
Department of Biology
The University of Western Ontario
2012-2013

Laboratory Analyst Intern
ALS Laboratories
Waterloo, Ontario
2010-2011

Publications:

Articles in Refereed Publications


Non-Refereed Contributions


Conferences: Oral Presentations (Presenter)


Conferences: Poster Presentations (Presenter)


