METAL ION BINDING OF METALLOTHIONEIN-2 CHARACTERIZED BY USING HIGH RESOLUTION NATIVE ESI-F-ICR MASS SPECTROMETRY

Touqeer Gill

Master's Thesis
Department of Chemistry Organic
Chemistry
607/2018
TABLE OF CONTENTS

TABLE OF CONTENTS ................................................................................................................. 1
ABBREVIATIONS ......................................................................................................................... 3
1. INTRODUCTION ....................................................................................................................... 4
1.1. Metallothionein (MTs) in general ......................................................................................... 4
  1.1.1 MTs Isoforms .................................................................................................................... 5
  1.1.2 Classification of MTs ......................................................................................................... 6
  1.1.3 Properties of Metallothionein .......................................................................................... 7
  1.1.4 Synthesis of Metallothionein ......................................................................................... 7
  1.1.5 Functions of Metallothionein ......................................................................................... 8
1.2. STRUCTURAL STUDIES OF METALLOTHIONEIN .............................................................. 10
  1.2.1 Spectroscopic Characterization ...................................................................................... 10
  1.2.2 MTs Structural Determination by Optical Techniques: Circular Dichroism, Ultra Violet Emission and Ultra Violet Absorption Spectroscopy ......................................................................................... 11
1.3. Metal ion binding studies of metallothioneins ..................................................................... 12
  1.3.1 Proposed metalation pathways for metallothionein ........................................................ 14
1.4. Kinetic study of MTs for Metalation and Demetalation ......................................................... 16
1.5. Mass Spectrometry ............................................................................................................... 17
  1.5.1 Introduction to Mass spectrometry ................................................................................ 17
  1.5.2 Native Mass Spectrometry ............................................................................................. 18
  1.5.3 Buffer Exchange ............................................................................................................. 18
1.6. Electro spray Ionization (ESI) ............................................................................................. 19
1.7. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS) ................ 20
  1.7.1 Components of FT-ICR MS instrument ....................................................................... 21
1.8. Tesla solariXTM FT ICR mass spectrometer ....................................................................... 23
2. AIMS OF THE STUDY .............................................................................................................. 23
3. MATERIAL AND METHODS .................................................................................................... 24
  3.1. Protein and Peptide Materials .......................................................................................... 24
  3.2. Electrospray ionization mass spectrometry (ESI-MS) ....................................................... 25
    3.2.1. Denaturating and native condition ............................................................................. 25
    3.2.2. MS Titration experiment ............................................................................................ 25
3.3. Mathematical model to calculate binding affinity ................................................................. 25
4. RESULTS .................................................................................................................................. 27
  4.1. Native & Denaturating ESI-MS of MT-2 ......................................................................... 27
4.2. Scrutinize the MT-2 Sub isoforms ................................................................. 29
4.3. MS binding titration ......................................................................................... 32
4.4. Metallothionein binding with other metals ..................................................... 33
5. DISCUSSION ........................................................................................................... 36
5.1 Scrutinize the MT-2 Sub isoforms ................................................................. 36
5.2 MS binding titration by 1, 10-phenanthroline ................................................. 36
5.3 Metals binding to apo-Metallothionein 2a ....................................................... 36
6. REFERENCES .......................................................................................................... 37
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>AES</td>
<td>Atomic emission spectroscopy</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>CAD</td>
<td>Collisional activated dissociation</td>
</tr>
<tr>
<td>CI</td>
<td>Chemical Ionization</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPP</td>
<td>Differential pulse polarography</td>
</tr>
<tr>
<td>DF</td>
<td>Double focusing</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray absorption fine structure electrospray</td>
</tr>
<tr>
<td>ESI MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FTICR-MS</td>
<td>Fourier transform ion cyclotron resonance mass spectrometry</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HEN</td>
<td>High efficiency nebulizer</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively coupled plasma</td>
</tr>
<tr>
<td>ICP–SFMS</td>
<td>Inductively coupled plasma–sector field mass spectrometer</td>
</tr>
<tr>
<td>ICP-TOF-MS</td>
<td>Inductively coupled plasma time of flight mass spectrometry</td>
</tr>
<tr>
<td>ICR</td>
<td>Ion cyclotron resonance</td>
</tr>
<tr>
<td>IRMPD</td>
<td>Infrared multiple photons dissociation</td>
</tr>
<tr>
<td>LMCT</td>
<td>Ligand to metal charge transfer</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MLPs</td>
<td>Metallothionein-like proteins</td>
</tr>
<tr>
<td>MALDI m/z</td>
<td>Matrix Assisted Laser Desorption Ionization</td>
</tr>
<tr>
<td>NMR</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PDB</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ROS</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPC</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RF</td>
<td>Reverse phase chromatography</td>
</tr>
<tr>
<td>SEC</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>TNF</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>TOF-MS</td>
<td>Tumor necrosis factor</td>
</tr>
</tbody>
</table>

Time-of-flight mass spectrometry
METAL ION BINDING OF METALLOTHIONEIN-2 CHARACTERIZED BY USING HIGH RESOLUTION NATIVE ESI FT-ICR MASS SPECTROMETRY

Touqeer Gill

Supervisors: Prof. Janne Jánis, MSc. Senthil Thangaraj, University of Eastern Finland, Department of Chemistry

University of Eastern Finland, Department of Chemistry, 2018

ABSTRACT

Metallothioneins (MTs) are intracellular, low molecular mass and cysteine-rich proteins and lack of aromatic amino acid residues. MTs have several interesting biological roles associated with the protection against DNA damage, oxidative stress and apoptosis. In this study ESI-FTICR mass spectrometry has been proven a powerful technique for the characterization of proteins in general directly from solution. A metallothionein experiment was conducted in rabbit-liver MTs, use of this technique allowed the identification of different sub isoforms of MTs (major: MT-2a, minor: MT-2b, MT-2c MT-2d and MT-2e) moreover traces of non-N-acetylated MT-2a and MT-2c were also observed in the form of minor peaks. Estimation of the zinc binding affinity with MT-2 performed by 1, 10-phenanthroline. Gradual increase of 1, 10-phenanthroline concentration demetallated Zn7-MT-2 and exhibit multiple peaks indicative of the binding of a second, third and fourth 1, 10-phenanthroline molecule to the complex and the simultaneous build-up of apo-MT-2. Fitting of the Zn7-MT-2 demetallation curves performed by using the Hill equation. Remarkable metal binding property of MT-2 have been reported that was assessed by using a variety of other alkali and transition metals. Metallothionein binding with other metals reveals that MT-2 was able to bind Ag⁺, and Co²⁺ but with low affinity. MT binds with both metals specifically such as Ag⁺ shows one, two, four, five, six and seven times binding whereas Co²⁺ reveal one, two, three, five, six- and seven-times binding. As a conclusion of this study and experiments we found out that Hg²⁺ ions were seen to bind with the protein from four to seven times and only gold Au³⁺ and Cooper Cu²⁺ were able to bind with the MT-2 protein. Hence the binding of the gold and copper ion to the MT-2 protein exhibit one, two, three, four, five, six- and seven-times binding.
1. INTRODUCTION

1.1. Metallothionein (MTs) in general

Analytical methodologies developed that study bioavailability as well as ecological impression of metalloids on existing organisms proposed ongoing experiments for enviromental chemistry and clinical toxicology.[1]

Metalloproteins take part in many biochemical processes. Metallothionein (MT) is a distinctive metalloprotein which 35 years of investigation still awaiting for its complete functional characterization.[2] In 1957 Margoshes and Vallee first discovered MT during the metal interating protein study that is the cause of cd accertion inside the cortex of kidney.[3] The mammalian form of MTs establishes a superfamily of ubiquitous cytosolic.[4] Metallothionein’s are metalloprotein have molecular masses 6-7 KDa contains 61-68 amino acid and 20 cysteine residues coordinated to 7 Zinc or Cadmium ions through metal-thiolate bonds and complete removal of aromatic residues.[5][6]

MTs proteins exist inside the cells whereas in higher organisms it occurs in extracellular media for instance cerebrospinal fluid and blood plasma.[7] Because of its flexibility and cysteine rich content, help them to adopt many conformations, and this exceptional phenomenon of elasticity allows MTs to form metal thiolate clusters with different metals. Moreover, lacking stiffness in MTs structure allows it to make coordination complexes with different metals like Cu\(^{2+}\) and Zn\(^{2+}\), virulent metals for instance Hg and Cd, Ur and Tc. These exceptions in metallothionein function, makes it more hard to analyze properly.[8]

Metallothionein establish polymorphic systems therefore its isoforms play beneficial biological roles in almost all organisms.[9] Metallothionein’s are resistant to thermos coagulation and acid precipitation. Metallothionein’s have exciting class of proteins from both inorganic and biochemical point of view because of their binding affinity with group 11 and 12 transition metals.

Mammalian MTs are the most predominant that are sequestered from brain, kidney and liver as well as they are also isolated from plants, mussels and Fungai.[10] They are vastly polymorphic protein demonstrating very low sequence similarity.[11] Rabbit liver MTs full chain have two domain □ and □- □- domain. figure 1 presented the Rabbit Liver MT-2 □ and □-domain along sequence of amino acid.[3]
Physico-chemical properties of MTs have been examined. Electrochemical and Immunochemical method such as differential pulse polarography and radioimmunoassay are used by Biochemists as conventional methods for the exploration of metallothionein. But still there is deficiency in analytical techniques with high accuracy and precision for better assessment of MTs.

1.1.1 MTs Isoforms

Being the product of genetic polymorphism, metallothionein has different isoforms which can be separate and analyze by an ion exchange chromatography technique. These isoforms are further classified into different sub isoforms on the basis various factors for example their molecular mass, atoms binding pattern, chromosomes, amino acid sequence and environment or metal binding properties. Separation of these sub isoforms demands high-resolution techniques such as mass spectrometry.

Hubert Chassaigne et al. studied the phenomenon of polymorphism in rabbit liver MTs and its major iso-forms such as MT-1 and MT-2 by reversed phased HPLC along with mass spectrometry. During this study each peak from assay analyzed as single chromatographic peak of metallothionein and their molecular mass were optimized by ion spray mass spectrometry.
There are four major isoforms of metallothionein designated as MT-I to MT-IV. The extensively spread isoform are MT- and MT-2 which exists in almost every tissues and organs, including brain, whereas MT-3 and MT-4 are less spread isoforms and occurs in central nervous system, squamous epithelium, that is a protective tissue and occur on many organs like skin, tongue, and vagina.\[19] \[20]

V. Sanz Nebot et al.\[21\] in their work isolated the different isoform of rabbit liver metallothionein by using capillary electrophoresis enhanced by liquid chromatography. In addition, they characterized these separated isoforms by Inductively coupled plasmas sector field mass spectrometry.

The vital challenges in MTs analysis include (1) original structure preservation of Metallothionein as well as metal complexes protection from devastation, (2) The interference of matrix elimination also sustain sensitivity for the instrumental recognition, (3) obtain metal components evidence in metal MTs complexes, and (4)MTs isoforms and sub isoforms separation and identification.\[22\]

Xu Guo1 et al. examined rabbit liver metallothionein moreover identified (MT-2C, MT2A, MT-2D and MT-2E) these four isoforms out of six. Another peak obtained comprise MT-1A or MT-2B and having 1 Da molecular masses difference. Whereas in the case of rat liver metallothionein MT-1 and MT-2 isoforms settled and described.\[6\]

Rongying Wang et al. proposed very swift and easy method for the MTs isoforms existing in Human prostate cells based on MALDI-TOF/TOF-MS. In that work, MT isoforms were identified by high mass accuracy and precision as well as superior MS/MS spectra analyzed with MALDI/TOF.\[23\]

1.1.2 Classification of MTs

In the late 1900 Binz and Kägi proposed MT classification on the basis of taxonomy.\[11\] Historically metallothionein’s are classified in three groups on the basis of their sequence similarities. Recent studies have modified this classification of MTs in more detail with the help of computational study in which they are further distributed in families, classes, and sub classes depending upon the cysteine residue location. There are 20 highly wellmannered cysteines residues arranged in mammalian metallothionein of class I. The arrangement of these residues is like CXC in which C shows cysteine residue whereas X shows other amino acids gathered in N and C terminal region. In two metal clusters such as (Me(II))3Cys9 and (Me(II))4Cys11 seven divalent metals bounded whereas metal binding domains connected by flexible length. In contrast yeast metallothionein shows typically class II which contain 12 cysteines residues distributed and bound with eight Cu(I) ions in a single cluster throughout the sequence.\[24\] There are four different types of plant metallothionein such as MT-1 to MT-4 and they are classified based on cysteine residue distribution in their terminal regions (N and C). Metallothionein each type shows
different three dimensional and chronological pattern during development due to that reason they also have different functions.[25]

1.1.3 Properties of Metallothionein

Metallothionein has very different property such as most of the protein contain aromatic amino acid whereas in the case of metallothionein it is absent due to that reason it is free from the absorbance region range that is 230 and 300nm. This property of MTs is appreciated because metals binding to the metallothionein cysteine residues SH group absorbance from aromatic amino acids absorption mask the (LMCT) absorption.

From these LMCT bands maximum excitation shows that the bound metals can be identify quite precisely from the absorption spectrum, and circular dichroism spectrum. Metal ion binding property is the most unique property of the metallothionein in which metal ion interact with sulfur atom and becomes kinetically reactive labile and thermodynamically static which means continuous breach and regeneration of metal SH (Thiol) bonds within metal clusters.[26][27]

1.1.4 Synthesis of Metallothionein

Previously scientists had synthesized MT which was not as expected as there were changes in intrinsic properties due to the cysteine rich peptides. Alternatively, they proposed recombinant DNA approach which indeed gave the substantial extant of metallothionein which can be transformed into the desired metal complex form. Furthermore in 1990’s scientists led their experiment to synthesize mouse MTs in E. coli but only partial achievements were obtained because of polymorphic character of MT, this single eukaryotic protein in prokaryotic environment was showing limitations in its stability. Despite this fact, this synthetic protein was showing different satisfactory metallothionein expression in E. coli which encouraged them to explore it further. Induction in the synthesis of MTs had happened by different heavy metal ions for instance copper, zinc and cadmium as well as different vitamins, hormones, antibiotics and organic cytosis agents.[28][29]
**Figure 2.** An experimental recombinant DNA approach showing in vivo (Zn-MT, CdMT, and Cu-MT) and in Vitro (Cd-MT and Cu-MT) synthetic pattern.\[29\]

### 1.1.5 Functions of Metallothionein

MTs have been sanctioned with many significant and crucial functions. MTs different isoforms have varieties of functions in cellular system such as MT-1 and MT-2 perform treatment of substantial and non substantial heavy metals for example Cd and Hg homeostasis assure Zn\(^{2+}\) and Cu\(^{2+}\) ions to the apoenzymes, metal channelize response, free radicals for instance reactive oxygen species, reactive nitrogen species scavenging and DNA damage, in animals, plants, microorganisms and adaptation to stress because of its cysteinyl thiolate groups.\[30\]

Due to these functions, metallothionine becomes the strong identifier agent for metal exposure which also provides defence against xenobiotics. These metalloproteins are considered as multifunctional proteins because they are comprised of different cellular process such as providing protection against neuro degenerative diseases and apoptosis.\[31\]\[32]\[33\]
Figure 3. A graphic presentation of mammalian metallothionein at different cellular levels.\(^{[32]}\)

Chubatsu et al.\(^{[34]}\) explored the role of metallothionein for the protection of V79 Chinese hamster cells against oxidative damage. This study revealed that the addition of zinc in metallothionein induced the expression of Chinese hamster cells without increasing the GSH level. Furthermore, these cells were providing the resistance in the production of DNA-strand scission.\(^{[35]}\)

Metallothioneine are also involved in different ailments such as MT-3 was discovered unexpectedly from the patient’s brain suffering from Alzheimer’s disease. This MT-3 shows non-inducible function and playing important role in the neurophysiological and neuromodulator aspects. Despite of gaining this much of information, there are still a lot of indescribable biological and environmental factors of metallothioneine left to discover which induced its synthesis and functions.\(^{[36]}\)

Roger S. Chung et al.\(^{[37]}\) studied the metallothionein expression in Alzheimer’s disease AD in rat cortical neurons with cooper and zinc binding. This study concluded that AD occurs when extracellular □-amyloid gets deposited in the cell without changing its state. They explained that MT-2A, can prevent this problem by swapping Cu (II)-A□□ with Zn\(_7\)MT-2A. In addition, MT-2A can protect against A□ aggregation and toxicity.
The isoforms of metallothionen known as MT-2A discovered to involved in cell proliferation and leads to the breast cancer. In addition to this MT-1E and MT-1F recognized as actively participating in breast cancer by alternating the estrogen level. Kelly et al. and Naganuma et al. suggested that cystein residues alkylatation in metallothionein perform resistance against anticancer therapeutics.

1.2. STRUCTURAL STUDIES OF METALLOTHIONEIN

1.2.1 Spectroscopic Characterization

The three-dimensional mammalian MTs protein structure was reported by NMR Arseniev et al., whereas in 1991 Robbins et al., proposed its X-ray crystallographic structure. Spectroscopic characterization of fully metalated metallothionein has given evidence at atomic level. It describes that this protein is having dumbbell shape structure which consisted of two metal centered cluster. The N-terminal presented Cd$_3$(Cys)$_9$ along three bounded bridging sulfur atoms (denoted as β-domain) while C-terminal presented Cd$_4$(Cys)$_{11}$ with five bridging sulfur atoms (α-domain). Introduction of chelating agent or acidification and removal of metal ions can collapse the 3D structure of MTs. Polymorphic character of metallothionein structure makes the structure of Cd-MT-1 to Cd-MT-3 flexible due to that reason it becomes very difficult to analyze its isoforms properly.

Metallothionein partially metalated structures is effective by NMR at lower pH, cooperative cluster formation produces sharp peaks whereas at higher pH it shows broad undistinguished peak. Hence, after the formation of metal thiolate cluster only NMR techniques are appropriate. To acquire a well depiction of metallothionein structure, more outlandish techniques must be used.
Hathout et al.\textsuperscript{[44]} characterized the two states of metallothionein for instance stable and transient dimeric state. They used the previous study data like NMR, SE chromatography and naonospray MS to resolve this myth. During this study metal ion quick redistribution between Cd\textsuperscript{7–} and Zn\textsuperscript{7–} metallothionein 2a was scrutinized with naonospray MS whereas the reconstructed Zn\textsuperscript{7–} or Cd\textsuperscript{7–} metallothionein exposed the existence of monomeric and dimeric classes by SE chromatography.

There are certain difficulties in x-ray diffraction (XRD) and nuclear magnetic resonance (NMR) analysis and it is very hard to get comprehensive three-dimensional structural data of metallothionein. Therefore, any minor evidence related to MTs is highly noteworthy.\textsuperscript{[45]}

1.2.2 MTs Structural Determination by Optical Techniques: Circular Dichroism, Ultra violet Emission spectroscopy and Ultra Violet Absorption spectroscopy.

Characterization of MTs and its metal interaction has done with variety of optical techniques. Metallothionein having absence of aromatic amino acid that limits use of UV-visible spectroscopy. \textsuperscript{[46][47]}

Circular dichroism technique used to observe the more sensitive changes in the symmetrical structure of SH (Thiol) cluster with metal atom and changes occur when the coupling takes place at its excitation level. This exciton coupling shows the Cd-thiolate LMCT band maximum absorption at 250nm crossover point. Disrupt coupling of MTs such as super or partially metallated MTs leads the shift of absorption maximum from 250 nm till 240–245 nm that is its crossover point.\textsuperscript{[48][49]}

\textbf{Figure 5.} Metallothionein dumbbell shape molecular model with two distinct metal centered cluster. Red bigger beads (Red color) representing metal ions such as Zn or Cd, while smaller one showing atoms of sulfur.\textsuperscript{[35]}
Most of these techniques are restricted when lots of metal atoms binds to the metallothionein structure resolution the reason behind this is that they provide limited answer for entire types that are present in sample. Moreover, few methods are limited for spectroscopic hushed Zn and also biologically appropriate metals that interact with metallothionein. The importance of mass spectrometry reveals here because it differentiates every species that is occurring in the sample also quantify their relative abundance. In addition, different techniques can provide MTs structure understanding at different stages such as Ion-mobility MS, tandem MS, residue modification MS.

1.3. Metal ion binding studies of metallothioneins

Folding of protein relying on metal scrutinized when single or multiple metal atoms binds with protein and give its fully functional conformation. One good example of metaldependent protein folding is the Cd-dependent folding of MT because they coupled with variety of metal atoms both in vivo and vitro states figure 6. MT interact with different metals from group 11 to 12 (Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Cu$^{+}$, Ag$^{+}$, and Au$^{+}$) as well as Fe$^{2+}$, Co$^{2+}$ and even Pb$^{2+}$. The metal interaction with MTs takes place by cysteine SH (thiolate) groups that are present at terminal as well as at bridging position and it is also observed that no other amino acid of MTs takes part in metal interaction.[50]

Metallothionein’s show a more binding interaction with Cd(II) as compare to Zn(II), such that even Cd scarcely dispersed in the environment will result in Cd accumulation in MTs. The binding constant of Cd$^7$-MT2a is 3.2 × 10$^{17}$ M$^{-1}$ at pH 7.4 whereas Zn$^7$-MT2a having Kd value 3.2×10$^{13}$M$^{-1}$ at pH 7.4. Although Cd(II) exhibit10,000 times more binding interaction with MT as compared to Zn$^{2+}$ that means ingested Cd$^{2+}$ will displace Zn$^{2+}$ in Zn-MT.[51]

![Figure 6. Metal-dependent protein folding of metallothioneins in vitro. (B) Red circles represent Cd ions, which induce the synthesis of MT. (C) Metallation starts in the α domain binding up to four Cd, then the β domain. (D) Seven Cd ions bind to MT; the α domain consists of four Cd and the β domain consists of three Cd.[51]](image)
Bin Hu et al. [20], Maria Montes Bayon et al. [52] and Dirk Schaumlöffel et al. [13] scrutinized the metallothionein by hyphenated techniques of mass spectrometry and characterize the metal complexes of MTs also express the binding interaction of metals with MTs. This evidence is enough to understand the toxicity mechanism caused by metal based nanomaterial to organism.

Gordon W. et al. [8] and Thanh T. et al. [53] recently, studied the residue modification of metallocothionine protein to probe its metalation properties by mass spectrometry. In this study, they concluded that to recognize the apo-MT dynamics of the conformers and its associated species different cysteine specific alkylation reagents can be use along with time dependent ESI-MS and step wise snapshot electrospray ionization MS.

Development of explicit coordination pattern happened when polypeptide chains bind firmly with metal ions and formation of systemized metal SH (Thiolate) cluster takes place. The 3D structures designed not only depending on the interaction of metal ions with protein rather it depends that how these metals are preferably binding with protein such as trigonal, or diagonal and tetrahedral manner. Characterization with EXAFS spectroscopic technique reveals Ag(I) may bind primarily with diagonal coordination rather than the trigonal coordination of Cu(I)-MTs. [54]

Zinc binding to MTs proved that it is physiological activity. By several studies, it has been proved that Metallothioninein performs as a chaperone of zinc and regulate the activity
of the protein such as gene expression moreover regulate the transcription factor of metallothionein relying on its metal interaction representing by figure 8. Thermodynamically zinc binding to Metallothionein is very steady that shows in vivo metallothionein is perfect Zn reservoir.

Figure 8. Metallothionein functions and gene regulation overview.[35]

Maret et al.[55] studied that exchange of Zn is faster in isoform 1 and 2 of metallothionein. Furthermore, Jacob et al.[56] proved that exchange of Zn is possible in metallothionein and Zn proteins apo-forms such as bovine carboxypeptidase A and E. coli alkaline phosphatase.

Consecutive additions of metal ions, that has considered by polarographic techniques is arising the evolution of different metallothionein species and its kinetics. Characterization of MT shows distinguish polarographic peaks of metal ions by the use of differential pulse polarography spectroscopy however it is in free state or interacting with MT and forming one cation pure complex with protein or mixed complex with more than one metal ions. A similar performance observed in the presence of cadmium or zinc along individual apo and apo-MT domains. These results are sanction by the use of Mass spectrometry.[57][58][59]

1.3.1 Proposed metalation pathways for metallothionein

Two metalation trails have been anticipated for metallothionein such as cooperative pathway and non-cooperative pathway. These trails do not have the similar behaviors due to different aspects for example binding pattern of metal ions towards the MTs. In cooperative binding pattern formation of stable metal cluster happens when metal interact first with the terminal SH (Thiol) group after that with bridging thiols figure 9.
Terminally bound intermediates are less dominant in this pathway. It was reported that the first four Co ions bind to terminal thiols of rabbit liver metallothionein 1 in a tetrahedral geometry prior to the formation of Co-thiolate clusters. When divalent metals binds to the seven metal bindings sites on MT, metal binding starts at the ∆ domain establish very constant metal cluster in a cooperative manner, while β domain follows the cooperative manner but forming a less stable cluster. However, demetallation follows the reverse order of metalation; the β domain releases its metals prior to the α domain. In non-cooperative pathway each metalation process does not depend on each other and take place individually at each domain. Metallothionein non-cooperative trail proceed by the addition of metal ion at terminal thiol (SH) group and bead like formation will occur. In this mechanism metal cluster with four metal atoms are less obvious but intermediate can be seen clearly.\[51\]

Devika P. Jayawardena predicted that at low pH, MT-2a will show a cooperative metal binding pathway whereas, at physiological pH, MT2a will show a non-cooperative metal interaction pathway. Less pH destabilizes the formation of individual bead-like structures and favors the formation of clusters.\[51\]

**Figure 9.** Two proposed metallation pathways of MT for divalent metals; Zn$^{2+}$ binding to MT-2a is used as an example. The cooperative (cluster formation) pathway is shown following pink arrows (D & E) and non-cooperative (beads formation) pathway is shown
following blue arrows (B & C). In the non-cooperative pathway, 20 cysteine residues are terminally bound to five metals, and the 6th and 7th metal bind to form two stable clusters. In the cooperative pathway, four metals bind to 11 cysteine residues forming a stable M₄S₁₁ cluster (D) and then three metals bind to nine cysteine residues forming two stable clusters (F). The intermediates (Zn₁-MT2, Zn₂-MT2, and Zn₃-MT2) are dominant in the spectrum is shown at the top (beads formation) and clusters (Zn₄-MT2) are dominant in the spectrum is shown at the bottom (cluster formation).[^51]

1.4. Kinetic study of MTs for Metalation and Demetalation

MT kinetic studies for metalation and demetalation pathways have shown very fascinating results because of fast reaction rates mechanism was not elucidated clearly. Recent As³⁺ interaction study made it somehow easier. Phenomenon of metalation occur when metal ion exchange from fully metallated metallothionein or interact with the apoMT. For the demetalation of metal ion from MT chelator or an electrophile is use. Meloni et al. research studies explain that formation of SDS aggregates can be prevent when Zn(II)-MT-3 isoform replace the metal ions from Cu(II)-Å.[^37]

Shu-Hua Chen et al. investigated MT-2A metalation and demetallation by Cd²⁺ for this study they adopted chemical labeling approach along mass spectrometry and revealed that this mechanism is domain specific and take place with two different manners.[^43] during the metalation process Cd²⁺ ions addition happens sequentially that present an intermediate (Cd₄MT) whereas removal of Cd²⁺ ions take place from β-domain and then from α-domain with the help of chelator such as EDTA.

![Figure 10. Metallothionein 2A metalation and demetallation pathways with Cd²⁺[^43]](Figure 10. Metallothionein 2A metalation and demetallation pathways with Cd²⁺[^43])

Munoz et al. and Petering et al. were considered apo-MT metalation with Cd²⁺, Zn²⁺, and Co²⁺.[^22] In this work, they studied apo-MT metalation with Zinc and cadmium at neutral pH (7) that was performed very quickly around 4ms. From this metalation work
they determined that binding of Co\textsuperscript{2+} initially happened accidentally and then metal thiolate cluster formation occurred by rearrangement.

There are variety of spectroscopic techniques like atomic absorption, emission and ICP spectroscopy and mass spectrometry that are used to characterize biological samples and identify the existence of even minor detail of metals in it. In these analysis pure sample is used.\textsuperscript{[60]} Moreover, these classical methods provide complete information of MTs isoforms or sub isoforms. The functional properties, dynamics and structure of metalloproteins analyze by emerging ES-MS due to its successful achievements.\textsuperscript{[20]}

1.5. Mass Spectrometry

1.5.1 Introduction to Mass spectrometry

Since its development in 1912 by physicist J.J. Thomson, mass spectrometry has become an influential analytical tool for providing molecular weight information, and for interpreting the structural details of both small and large molecules. Mass spectrometer has been known as significant instrument to characterize Post Translational Modification PTMs due to its sensitivity, accuracy, selectivity, speed, and low consumption of samples. Modified proteins can be simply analyzed by MS due to the mass shift arising from the specific modification, and the modification site can be further localized by tandem MS.\textsuperscript{[61][62]}

There are three main components in a mass spectrometer, including the source of ionization, detector and mass analyzer. Fundamentally, mass spectrometry (MS) comprises on to generate the ions in the gas phase from desired sample, ions separating related to their mass to charge ratio (m/z), after that detect relative intensities of the individual ionic species. A computer stores the data and generates a mass spectrum, in which x-axis presented m/z whereas y-axis represent abundance of ions. From a mass spectrum, it is possible to gain both quantitative and qualitative information about complex chemical and biological mixtures, with masses ranging to over 4 MDa.\textsuperscript{[63][64]}

Before 1950, MS was only used for qualitative and quantitative analysis of different organic compounds and only suitable for small (<1000 Da), volatile compounds. After that chemists began to understand the fragmentation mechanism that occur within the mass spectrometer.\textsuperscript{[65][66][67]} At that time the most old style ionization method electron impact (EI) ionization was using although later on mild ions dissociation technique such as MALDI and ESI were implemented that made mass spectrometry a viable instrument for studying large biological systems.\textsuperscript{[68][69][70]}
1.5.2 Native Mass Spectrometry

Mass Spectrometry is densely used in the pharmaceutical industry for the characterization of biological molecules and to measure the purity of proteins, monoclonal antibodies, as well as used in the proteomics field. In 1990, for the first time, the non-denaturing or native MS was reported by two American groups who observed the intact protein metal-complexes in the gas state using an ESI-MS. Figure 11 shows MS spectra of the native and the denaturing conditions. The difference between native and denature condition is that native condition has comparatively few charged state distribution whereas the denature condition has more charge state distribution. The native state in mass spectrometric experiment have buffered solution at pH 7, 37°C and appropriate salt concentration along chemical denaturants absence.

![Figure 11. Mass spectra of a) denaturing state and b) the native state of protein H. pylori urease.](image)

1.5.3 Buffer Exchange

Buffer is responsible in creating changes in mass spectrometry analysis due to the signals generated by salt present in it as well as adducts formation in desired sample. To prevent these changes in MS analysis, buffer must be volatile which leads the sample to change into gas state completely and on the other hand it will minimize the generation of unwanted peaks. In the case of native mass spectrometry buffer needs to be highly...
rigorous, volatile and should not change the original state of protein sample. Moreover, in some cases could even use other non-volatile buffers. The good examples of buffer that are frequently in use for native mass spectrometry analysis are NH$_4$OAc (pH 7) and NH$_4$HOAc.[74]

Buffer exchange plays vital role in mass spectrometric analysis by upsetting the exact mass and induces the adduct formation (Na$^+$/ K$^+$) and high salt concentration. Therefore, the volatile buffers should be selected so that it should not conquer the ESI signals. Tris buffer stabilizes original metalloprotein complexes. To obtain the better picture from the analyte, concentration should be given to the sample preparation taking care of condition and parameter of ESI-MS instrument.

The important thing that needs to be taken care while scrutinising the protein analysis are to maintain the pH of sample solution and reducing the factors like salts and adduct formations that makes the sample non-volatile. If the oxidation occurs in the sample signal, then to suppress them use DTT as reducing agent.[10] ESI-MS analysis is the technique that permits the exact visualization of metal states (uptake, release, exchange and ions transfer) in protein sample.[73]

1.6. **Electrospray Ionization (ESI)**

The invention of electrospray ionization is attributed to the work of Malcolm Dole in the 1960s, however, it was, invented by Fenn in the late 1980’s, along with MALDI, are the two revolutionary soft ionization techniques, which make sure the analysis of large bio molecules. In the year of 2002 John B. Fenn and Koichi Tanaka jointly received the nobel prize for the evolution of soft ionization technique along MS for the identification and structure analyses of bio molecules.

**Figure 12.** Electrospray ionization (ESI) setup. Positive charges are represented as pink circles, negative ions as green circles, and solvent molecules as blue circles.[75]
A typical electrospray ionization setup is shown in figure 12. In electrospray ionization study analyte is first properly mix in buffer media and then introduced into a metal capillary tube. The high potential (2-6 kV), in combination with a surrounding nebulizing gas, aids in drawing the sample solution out of the small opening of the capillary and breaks it into charged droplets on the order of several microns in diameter.

A counter current flow of dry gas is often used to support the evaporation of droplets and a dry N₂ gas is used to diminish the radial dispersion of the spray. Rayleigh limit is the phenomenon in which coulombic repulsion force gets attach to the surface tension of the droplets at that point droplet undergoes coulombic burst, or fission, producing smaller, charged daughter droplets, which subsequently undergo further evaporation. Repeated events of evaporation and fission ultimately lead to droplets of nanometer size, from which analyte molecules are released as intact gas phase ions method \((\text{M}+n\text{H})^+\) (in +Ve ion form) or \((\text{M}-n\text{H})^-\) (in -Ve ion form), which are drawn into the mass spectrometer by the potential and pressure gradients. Multiply charged ions are often formed that is the plus point of electrospray ionization, that leads to improve the sensitivity, and allows the analysis of high molecular weight molecules. Furthermore, ESI is a soft ionization method so the whole molecules are observed. All in all, it can use to the analyze the variety of samples, such as peptides, proteins, organometallics, polymers, petroleum, organic compounds, etc.[76][77][78][79][80]

1.7. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR-MS)

1930 Ernest o. lawrence discovered the cyclotron resonance acceleration theory in 1930s and later on in 1950s it was first applied into a mass spectrometer by Sommer et al. Melvin B. Comisarow and Alan G. Marshall implemented FT method which was having ion cyclotron resonance and further used after two decades to build first FTICR-MS instrument. The FT-ICR in a uniform magnetic field regulates m/z value of ions by computing their frequency of cyclotron.[81]

Trapping of ions take place in both homogenous axial magnetic and electric filed as shown in figure 13. Ions resonantly jump from ground state to the excited state when resonance frequency apply towards the two ions exciting plates. The coherent ion cyclotron signal of an ion by given m/z induces charge image in the detection plates. The image charge is converted, enlarged, and digitized to produce the time domain transient. Transient signal can be Fourier transformed into a frequency domain spectrum, calibrated, and changed into MS spectra. FT-ICR-MS profit are that the excited ions can travel a distance of kilometers during a detection time of a 1⁷. Hence, for a typical transient length of a few seconds, a resolving power over \(~10^6\) can be attained.[82][83][84]
1.7.1 Components of FT-ICR MS instrument

There are some main components of all FT-ICR instruments which take part in its working magnet, a mass analyzer ion cyclotron resonance cell (ICR cell), ultrahigh vacuum system, and data system. The magnet which is used including permanent magnets, electromagnets, and superconducting magnets, actively shielded superconducting magnets are the most commonly used ones. The core part of the ICR cell is introduced in detail in the following section, along with ion motions in the ICR cell, excitation, and detection.\textsuperscript{[70]}

1.8. Tesla solariXTM FT ICR mass spectrometer

First ions are generated using either the ESI/MALDI source, then passed through the heated glass capillary and move towards the ion funnel and skimmer region. The double
ion funnels can efficiently emphasis ions and transfer them more effectively. Ions are then focused in the source octapole before passing through the quadrupole and hexapole collision cell. Once performing collisionally activated dissociation (CAD), Quadrpole separate the ions while collisional hexapole cell unit accumulate and fragmented them. After that ions fly through the hexapole and enter into the infinity cell where ECD and IRMPD achieved.

Determining the power of mass at m/z 400 over 500, 000 with mass accuracy < 0.5 ppm with internal mass calibration, and < 2 ppm with external calibration can be attained by using this 12T solariX FTICR MS.^[70]
2. AIMS OF THE STUDY

In this research, we have established a native MS based method for metallothionein quantification binding to zinc metal atom using high resolution Fourier Transform Ion Cyclotron Resonance (FT-ICR) Mass spectrometry coupled with electrospray ionization (ESI) source. More specifically, we have focused on the following topics,

1. The characterization of metallothionein in denaturing and native-like condition.
2. We have developed a methodology to study the metal ion binding and cysteine redox status upon oxidative stress with the use of ESI FT-ICR mass spectrometry. In particular, we have used DTT for the reduction of cysteine residue and used H$_2$O$_2$ for the oxidation.
3. We have performed the demetallation of MT-2 using 1,10-phenanthroline under optimal condition and identified the different isoforms of MT-2.
4. The binding affinity studies to quantify the fully zinc metalated MT-2 using metalspecific chelator 1, 10 phenanthroline with titration experiment.
5. Analysis of binding of MT-2 to different metal ions like (Ag$^+$, Au$^{3+}$, Co$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, and Zn$^{2+}$) was studied.
3. MATERIAL AND METHODS

3.1. Protein and Peptide Materials

The synthetic human metallothionein (MT-2; β domain) and rabbit liver (α & β domain) were purchased from GeneCust (Dudelange, Luxembourg) and Enzo Life Sciences (Gammel, Denmark) as a lyophilized powder (≥ 95% purity) (Table 1). The protein samples were dissolved in 20 mM ammonium acetate buffer comprising 20 mM DTT, pH 6.8, at a concentration of 0.1 mg.mL$^{-1}$. The stock solutions were stored at -18°C and, before the mass spectrometric experiments, was diluted to the desired final protein concentration and supplemented with 1 mM DTT. 1, 10-Phenanthroline (1,10-phen), dithiothreitol (DTT), ammonium acetate (≥ 99.999%), acetonitrile, water and acetic acid (99%) were best (HPLC grade) and acquired from Sigma Aldrich (HPLC grade). Hydrogen peroxide ($H_2O_2$) was received from Fulka analytical. The metal ions such as Zinc (Zn$^{2+}$), Cadmium (Cd$^{2+}$), Copper (Cu$^{2+/+}$), Silver (Ag$^+$), Mercury (Hg$^{2+}$) were received from Sigma-Aldrich whereas Gold (Au$^{3+/+}$) and Cobalt (Co$^{2+}$) were purchased from Alfa Aesar and all the metals were as an acetate form. Standard solutions of the all metal ions were prepared in water solution, at a concentration of ~100 mM.

Table 1. The sequence of MT-2 human and rabbit liver

<table>
<thead>
<tr>
<th>Metallothionein</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (β domain)</td>
<td>MDPNCSCAAG$^{10}$DSCTCAGSCK$^{20}$KECKCTSC$^{20}$</td>
</tr>
<tr>
<td>Rabbit Liver (α &amp; β domain)</td>
<td>MDPNCSCAAA$^{10}$GDSCTCANSC$^{20}$TCKACKCTSC$^{30}$KKSCCSCCPP$^{40}$GCA KCAQGCI$^{50}$CKGASDKCSCCA$^{62}$</td>
</tr>
</tbody>
</table>

Demetalation:

The lyophilized MT-2 (α&βdomain) was dissolved in 20 mM ammonium acetate buffer contained 20 mM DTT, pH 6.8, at a concentration of ~0.1 mg.ml$^{-1}$. Prior to mass spectrometric measurement, the demetalation of MT-2 was performed by adding 1,10phenanthroline (1:2, v/v) and incubated the sample at 25°C for 45 minutes, gently vortexed, and centrifuge against 5-kDa molecular weight cut-off centrifugal concentrator (Amicon Ultra or Vivaspin2; Millipore) for 20 min. The concentrated sample was used for the mass spectrometric measurement.
3.2. Electrospray ionization mass spectrometry (ESI-MS)

All the mass spectrometric experiments were achieved with a 12T solariX™ XR hybrid Qh-FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an Apollo II electrospray ionization (ESI) origin operated with positive ions dissociation mode. The ultra-high vacuum (~10⁻¹⁰ mbar) was generated with two rotary pumps and four turbo molecular pumps. All the experiments were completed from m/z 200 to 4000. The needle thrust flow rate (2 μl min⁻¹) along dry nitrogen (4.0 L min⁻¹) gas that serves as the nebulizer gas (80°C, 1.0 bar) used for the sample infusion. The ions were collected in collisional cell for 0.9s whereas the time-of-flight was set at 1.2 ms, the instrumental parameters were adjusted for optimal detection of the protein signals and to prevent unintentional collisional activation of the protein metal ion complexes. The mass spectrum was calibrated externally using ESI tuning mix calibration mixture (Part no: G1969-85000; Agilent Technologies, Santa Clara, CA). The data acquisition was acquired by FTMS Control 2.0 software, and data was processed by using Bruker Data Analysis 4.2 or 4.4 software.

3.2.1 Denaturating and native condition

The denaturated MT-2 samples were prepared in a mixture of (MeCN: H₂O: HOAc; 49.5:49.5:1, v/v) and MS spectra were measured using FI-ICR ESI mass spectrometer to assess the amino acid sequence, homogeneity and purity of the sample. 5 µM of MT-2 was used for the MS measurement, for the native-like condition, 20 mM ammonium acetate containing 20 mM DTT (pH 6.8) was used.

3.2.2 MS Titration experiment

High-sensitive MS titration experiments were performed by chelation technique (1,10Phen) with a concentration from 20 to 800 µM, a blank between each concentration at a 5 µM of constant MT-2+Zn7 concentration. There were three replicates measured on each concentration. The mathematical model was derived for to calculate the binding constant. The calculation and curve fitting were performed using Origin software.

3.3. Mathematical model to calculate binding affinity

In this study, the Zn-binding affinity of MT-2 was calculated using ESI-MS spectra from titration experiment of Zn₇MT-2 with 1,10-Phenanthroline by fitting the fractional occupancy of Zn (I) in MT-2 (Y) with the free Zn (I) ion concentration using the following equation. Here we considered seven Zn (I)-binding sites in MT-2.
Where, $I_{Zn,MT-2}$ refer to the intensity of the Zn$_{0}$MT-2 in MS spectra. The free Zn (I) ion concentration was calculated using the binding affinity of 1,10-Phenanthroline ($5.09 \pm 0.01$ M).

The obtained sigmoidal binding curve was fitted linear and nonlinear with their corresponding Hill equation using Origin 8.1 (Origin Lab Corporation).
4. RESULTS

4.1. Native & Denaturating ESI-MS of MT-2

In denaturating condition, the MS spectra of MT-2 (β and ☐-domain) was obtained from m/z 200 to 4000 and it showed only at +4-charge state (Fig 15). The experimental monoisotopic mass of the Human MT-2 (2692.7708 Da) is matching perfectly with the amino acid sequence based theoretical mass (2692.6096 Da; C\textsubscript{105}H\textsubscript{168}N\textsubscript{33}O\textsubscript{43}S\textsubscript{10}) Table 2. No binding was observed at this condition; however, the cysteine residues were not fully reduced. To ensure the full reduction, we have performed different ratios of DTT by varying solvents, pH, incubation time, different temperatures and degasified with argon. Unfortunately, the human MT-2 (☐ domain) is not at all being fully reduced, and for the native condition it does not shows any signals. Moreover, according to the manufacturer the purity of the MT-2 was ~80 %. So, this could be also one of the main reason that why don’t get any signal on native sate. At this stage, we have thought to use the full length MT-2 (☐ and ☐ domain) in rabbit liver.

![Figure 15. ESI-MS spectrum of Human MT-2 (β domain) a) denaturat ed oxidized b) denaturated partially reduced c) the calculated mass spectrum.](image-url)

In denaturating condition, the mass spectrum of the MT-2 (α and β domain) was obtained at m/z 200 to 3000 with charge state distribution (CSD) 3+, 4+, 5+ and 6+ state (Table...
2). The experimental monoisotopic mass of the MT-2 (6566.73096 Da) is matching perfectly with the amino acid sequence based theoretical mass (6566.57638 Da, C\textsubscript{226}H\textsubscript{382}N\textsubscript{72}O\textsubscript{84}S\textsubscript{21}) (Fig 16) of the Zn\textsubscript{7}MT-2. So, Zn\textsubscript{7} binding was observed at this condition. Similarly, the mass spectrum measured in native like conditions also showed Zn\textsubscript{7} binding, the ESI-Mass spectrum are in the range of m/z 200 to 3000 (Fig 16) with charge state distribution (CSD) 3+,4+,5+ and 6+ state, accompanied with the mass increment of 61.999 Da, which is consistent with seven zinc metal ions. The experimental most abundant isotopic mass of Zn\textsubscript{7}MT-2 (6566.6788 Da, C\textsubscript{226}H\textsubscript{382}N\textsubscript{72}O\textsubscript{84}S\textsubscript{21}) agrees well with the calculated mass (6566.57638 Da, C\textsubscript{226}H\textsubscript{382}N\textsubscript{72}O\textsubscript{84}S\textsubscript{21}) (Table 2). Assuming removal of 15 protons upon zinc binding, 2 protons for each metal atom and 1 more proton in the metal between α and β-domain. A complete metalated MT-2 observed, which indicates of high affinity. In addition, we observed a different sub isoform of MT-2 in the native condition along with Zn\textsubscript{7}MT-2 (Fig 16).

![Figure 16. ESI-MS spectrum of Zn\textsubscript{7} MT-2 (rabbit liver; α & β-domain) in native condition (20 mM ammonium acetate with 20 mM DTT, pH 6.8; the inset shows the sub isoforms of MT-2.](image)

**Table 2.** The observed monoisotopic mass of Metallothionein-2

<table>
<thead>
<tr>
<th>Metallothionein</th>
<th>Condition</th>
<th>Theoretical (Da)</th>
<th>Experimental (Da)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Liver (β-domain)</td>
<td>Native</td>
<td>6566.57638</td>
<td>6566.67688</td>
<td>0.1005</td>
</tr>
<tr>
<td></td>
<td>Denature</td>
<td>6566.57638</td>
<td>6566.73096</td>
<td>0.1545</td>
</tr>
<tr>
<td>Human (α&amp;β-domain)</td>
<td>Denature</td>
<td>2692.60960</td>
<td>2692.77080</td>
<td>0.1612</td>
</tr>
</tbody>
</table>
perfectly with the amino acid sequence based theoretical mass (6566.57638 Da, C_{226}H_{382}N_{72}O_{84}S_{21}) (Fig 16) of the Zn\textsubscript{7}MT-2. So, Zn\textsubscript{7} binding was observed at this condition. Similarly, the mass spectrum measured in native like conditions also showed Zn\textsubscript{7} binding, the ESI-Mass spectrum are in the range of m/z 200 to 3000 (Fig 16) with charge state distribution (CSD) 3+,4+,5+ and 6+ state, accompanied with the mass increment of 61.999 Da, which is consistent with seven zinc metal ions. The experimental most abundant isotopic mass of Zn\textsubscript{7}MT-2 (6566.6788 Da, C_{226}H_{382}N_{72}O_{84}S_{21}) agrees well with the calculated mass (6566.57638 Da, C_{226}H_{382}N_{72}O_{84}S_{21}) (Table 2). Assuming removal of 15 protons upon zinc binding, 2 protons for each metal atom and 1 more proton in the metal between α and β-domain. A complete metalated MT-2 observed, which indicates of high affinity. In addition, we observed a different sub isoform of MT-2 in the native condition along with Zn\textsubscript{7}MT-2 (Fig 16).

**Figure 16.** ESI-MS spectrum of Zn\textsubscript{7} MT-2 (rabbit liver; α & β-domain) in native condition (20 mM ammonium acetate with 20 mM DTT, pH 6.8; the inset shows the sub isoforms of MT-2.

**Table 2.** The observed monoisotopic mass of Metallothionein-2

<table>
<thead>
<tr>
<th>Metallothionein</th>
<th>Condition</th>
<th>Theoretical (Da)</th>
<th>Experimental (Da)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Liver (β-domain)</td>
<td>Native</td>
<td>6566.57638</td>
<td>6566.67688</td>
<td>0.1005</td>
</tr>
<tr>
<td></td>
<td>Denature</td>
<td>6566.57638</td>
<td>6566.73096</td>
<td>0.1545</td>
</tr>
<tr>
<td>Human (α&amp;β-domain)</td>
<td>Denature</td>
<td>2692.60960</td>
<td>2692.77080</td>
<td>0.1612</td>
</tr>
</tbody>
</table>
4.2. Scrutinize the MT-2 Sub isoforms

MT-2 mass spectrum of in 20 mM \( \text{NH}_4\text{OAc} \) (pH 6.8) containing 20 mM DTT in presence of 1,10-Phenanthroline, a strong chelator, was used for the demetalation (Fig 17). The demetalation (Apo) of MT-2 was performed (see method section); the presence of sub isoform was identified and compared with previously report article, which explains that all cysteine rings are in their reduced form. The experimental most abundant isotopic masses of the major sub isoforms (major: MT-2a, minor: MT-2b, MT-2c MT-2d and MT2e) was compared with the calculated mass and their corresponding amino acids sequences are also listed in table 3.

![MT-2 Fully Metallated](image1)

![MT-2 Fully (apo)](image2)

**Figure 17.** The ESI-MS of complete conversion of fully metalated (Holo) in to demetalated (Apo) form of MT-2 in presence of 1,10-Phenanthroline as a chelator; the comparison of experimental and calculated most abundant isotopic mass of MT-2 in apo form.
Figure 18. ESI-MS of MT-2 sub isoforms present in (Apo) MT-2 (major: MT-2a, minor: MT-2b, MT-2c MT-2d and MT-2e).

Among the different isoforms, the N-acetylated MT-2a was the major isoform followed by N-acetylated MT-2b and 2c. There are some minor isoforms also present.
Table 3. Identification of Rabbit Liver (Apo) MT-2 sub isoforms

<table>
<thead>
<tr>
<th>Isoform</th>
<th>m/z = 5</th>
<th>Most abundant mass, Calculated (Da)</th>
<th>Most abundant mass, Experimental (Da)</th>
<th>Error (ppm)</th>
<th>Amino acid Composition</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Ac-MT-2a</td>
<td>1225.8</td>
<td>6124.2010</td>
<td>6124.4635</td>
<td>0.2625</td>
<td>Ac-M1D3P3N2C20S8A9T3G4K7Q1I1</td>
<td>C226H382N72O84S21</td>
</tr>
<tr>
<td>MT-2a</td>
<td>1217.4</td>
<td>6082.1909</td>
<td>6081.9635</td>
<td>-0.2274</td>
<td>M1D3P3N2C20S8A9T3G4K7Q1I1</td>
<td>C224H380N72O83S21</td>
</tr>
<tr>
<td>N Ac-MT-2b</td>
<td>1230.0</td>
<td>6145.2120</td>
<td>6145.4635</td>
<td>0.2515</td>
<td>Ac-M1D3P2N1C20S9A6T4G4K8E1Q1I1</td>
<td>C226H383N71O86S21</td>
</tr>
<tr>
<td>N Ac-MT-2c</td>
<td>1231.8</td>
<td>6154.2120</td>
<td>6154.4635</td>
<td>0.2515</td>
<td>Ac-M1D3P2N2C20S8A8T5G4K7Q1I1</td>
<td>C227H384N72O85S21</td>
</tr>
<tr>
<td>MT-2c</td>
<td>1223.4</td>
<td>6112.2010</td>
<td>6112.4635</td>
<td>0.2625</td>
<td>M1D3P3N2C20S8A8T4G4K7Q1I1</td>
<td>C225H382N72O84S21</td>
</tr>
<tr>
<td>N Ac-MT-2d</td>
<td>1243.8</td>
<td>6214.2810</td>
<td>6214.4635</td>
<td>0.1825</td>
<td>Ac-M1D3P2N1C20S9A7T3G3K8E1Q1I1R1</td>
<td>C229H392N74O85S21</td>
</tr>
<tr>
<td>N Ac-MT-2e</td>
<td>1249.0</td>
<td>6240.3330</td>
<td>6240.4635</td>
<td>0.1305</td>
<td>Ac-M1D3P2N1C20S8A7T3G3K8Q1I1E1R1L1</td>
<td>C232H398N74O84S21</td>
</tr>
</tbody>
</table>
### 4.3. MS binding titration

An ESI-MS titration approach was applied to calculate the binding attraction of Zn(I) in MT-2, this method was to monitor the titration of holo (metalated) to apo (demetallated) in the presence of competing Zn (I)-binding chelator (1,10-Phen). In the titration experiment, a fixed Zn\textsubscript{7}MT-2 (5 \(\mu\)M) concentration was used with increasing 1,10-Phen concentration from 20 to 800 \(\mu\)M, supplemented with 1 mM of DTT. In order to determine the binding constant, the free Zn (I) ion and the fractional occupancy of the Zn\textsubscript{7}MT-2 was calculated for each 1,10-Phen concentration from the MS spectra, and fitted with the free Zn(I) concentration in solution.

**Figure 19.** ESI-MS of Zn\textsubscript{7} MT-2 to 1, 10-phenanthroline at different concentrations (20\(\mu\)M, 50\(\mu\)M, 100\(\mu\)M, 200\(\mu\)M, 300\(\mu\)M, 400\(\mu\)M, 500\(\mu\)M, 600\(\mu\)M, 700\(\mu\)M, 800\(\mu\)M) *and ** refers Holo.MT and Apo-MT. Numbers refers ion charge state of respective.
4.4. Metallothionein binding with other metals

To characterize metal ion binding of the peptides, the experiments were attempted in ammonium acetate buffer solution was used beside metals ion such as (Ag⁺, Au³⁺, Co²⁺, Cu²⁺, Hg²⁺, and Zn²⁺) by using three molar ratios, such as 1:1, 1:10, 1:50. However, metal-free (apo) MT protein reoxidation occurred rapidly in the absence of zinc. Therefore, reduced with DTT, an ESI-MS compatible reducing agent. However, during prolonged storage times the protein were found to reoxidize even in the presence of DTT. However, as DTT can also chelate zinc ions, the use of excessive DTT was avoided in further experiments.
Figure 21. ESI FT-ICR mass spectra of (apo)MT-2 protein with 10-fold molar excess of a) Au$^{3+}$, b) Ag$^+$, c) Cu$^{2+}$, d) Co$^{2+}$, and e) Hg$^{2+}$ ions. The protein concentration was 6 μM in each. Numbers refers to ion charge state whereas * shows one, two, three, four, five, six, and seven times binding of respective.

A mass spectrum measured for (apo) MT-2 in the existence of 10-fold molar excess of metals ion such as (Ag$^+$, Au$^{3+}$, Co$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, and Zn$^{2+}$) ions showed a marked change in the CSD (4+ to 5+) as compared to the apo-protein and after metal binding in figure 21. The specificity of MT-2 to bind with different metals was assessed by using a variety of other alkali and transition metals. Small amount of the (apo) protein was observed in some samples. Metallothionein binding with other metals reveals that MT-2 was able to bind Ag$^+$, and Co$^{2+}$ but with low affinity. MT binds with both metals specifically such as Ag$^+$ shows one, two, four, five, six and seven times binding whereas Co$^{2+}$ reveal one, two, three, five, six and seven times binding. In addition, surprisingly, Hg$^{2+}$ ions were seen to bind with the protein from four to seven times. Only gold Au$^{3+}$ and Cooper Cu$^{2+}$ were able to bind with the MT-2 protein figure 21. The binding of the gold and copper ion to the MT-2 protein exhibit one, two, three, four, five, six and seven times binding. It shows
their abundant complex formation with the MT-2 protein. Judith S. et al. [22] suggested that MTs has a binding with copper (I) ion in a step wise manner by following the mixed cooperative and non-cooperative mechanism. In this study the, low and very high concentration of metal ions such as 1:1 and 1:50 times does not allow the proper binding. A mass spectrum measured for (apo) MT-2 in 10 times molar excess of metals ion such as (Ag⁺, Au³⁺, Co²⁺, Cu²⁺, Hg²⁺, and Zn²⁺) ions showed a marked change in the CSD (4+ to 5+) as compared to the apo-protein and after metal binding in figure 21. The specificity of MT-2 to bind with different metals was assessed by using a variety of other alkali and transition metals. Small amount of the (apo) protein was observed in some samples. Metallothionein binding with other metals reveals that MT-2 was able to bind Ag⁺, and Co²⁺ but with low affinity. MT binds with both metals specifically such as Ag⁺ shows one, two, four, five, six and seven times binding whereas Co²⁺ reveal one, two, three, five, six and seven times binding. In addition, surprisingly, Hg²⁺ ions were seen to bind with the protein from four to seven times. Only gold Au³⁺ and Cooper Cu²⁺ were able to bind with the MT-2 protein figure 21. The binding of the gold and copper ion to the MT-2 protein exhibit one, two, three, four, five, six and seven times binding. It shows their abundant complex formation with the MT-2 protein. Judith S. et al. [22] suggested that MTs has a binding with copper (I) ion in a step wise manner by following the mixed cooperative and non-cooperative mechanism. In this study the, low and very high concentration of metal ions such as 1:1 and 1:50 times does not allow the proper binding.
5. DISCUSSION

5.1. Scrutinize the MT-2 Sub isoforms

To study the presence of sub isoform in the Zn$_7$-MT2 protein, 1,10-phenanthroline was used to demetallate the MT-2 (bound with 7 Zn). Fractions that contained metal-free (apo) MT were analyzed by ESI-MS that showed evidence of MT-2 sub isoforms (major: MT2a, minor: MT-2b, MT-2c MT-2d and MT-2e) exhibit in figure 18.

5.2. MS binding titration by 1, 10-phenanthroline

For the estimation of the zinc binding affinity experiment with MT-2. The full titration of Zn$_7$-MT-2 was performed by 1, 10-phenanthroline that demetallated Zn$_7$-MT-2 at micromolar concentrations through a stepwise process. For that purpose, 20µM, 50µM, 100µM, 200µM, 300µM, 400µM, 500µM, 600µM, 700µM, and 800µM concentrations were used. Multiple peaks appeared at higher concentrations of 1, 10-phenanthroline, indicative of the binding of a second, third and fourth 1, 10-phenanthroline molecule to the complex and the simultaneous build-up of apo-MT-2 figure 19. The binding of multiple 1, 10-phenanthroline ions appeared to lead to the opening of Zn(II)-thiolate clusters and Zn$^{2+}$ ions dissociation from the protein. Sequential addition of 1, 10-phenanthroline caused the amount of Zn-MT2a to decrease, while the amount of apoMT2a increase. As dissociation of Zn(II) -thiolate clusters of Zn$_7$-MT-2 occurred cooperatively we did not observe hyperbolic demetallation curve. By this reason fitting of the Zn$_7$-MT-2 demetallation curves by the influence of 1, 10-phenanthroline was performed by using the Hill equation.

5.3. Metals binding to apo-Metallothionein 2a

Stepwise addition of different metals to apo-MT-2a showed evidence of bead-like structures with metals binding to terminal thiols, which is indicative of the noncooperative metal binding pathway figure 21. Sequential addition of approximately 2.0 molar equivalents of metals caused the amount of Zn-MT2a to increase, while the amount of apo-MT2a decreased. Apo-MT-2a readily picked up the metals between pH 6 and 7.
6. REFERENCES


[51] D. P. Jayawardena, **2017**.


[75] B. L. Heath, B. L. Heath, **2012**.


4.2. Scrutinize the MT-2 Sub isoforms

MT-2 mass spectrum of in 20 mM NH₄OAc (pH 6.8) containing 20 mM DTT in presence of 1,10-Phenanthroline, a strong chelator, was used for the demetalation (Fig 17). The demetalation (Apo) of MT-2 was performed (see method section); the presence of sub isoform was identified and compared with previously report article, which explains that all cysteine rings are in their reduced form. The experimental most abundant isotopic masses of the major sub isoforms (major: MT-2a, minor: MT-2b, MT-2c MT-2d and MT2e) was compared with the calculated mass and their corresponding amino acids sequences are also listed in table 3.

Figure 17. The ESI-MS of complete conversion of fully metalated (Holo) in to demetalated (Apo) form of MT-2 in presence of 1,10-Phenanthroline as a chelator; the comparison of experimental and calculated most abundant isotopic mass of MT-2 in apo form.
Figure 18. ESI-MS of MT-2 sub isoforms present in (Apo) MT-2 (major: MT-2a, minor: MT-2b, MT-2c MT-2d and MT-2e).

Among the different isoforms, the N-acetylated MT-2a was the major isoform followed by N-acetylated MT-2b and 2c. There are some minor isoforms also present.
4.3. MS binding titration

An ESI-MS titration approach was applied to calculate the binding attraction of Zn(I) in MT-2, this method was to monitor the titration of holo (metalated) to apo (demetalated) in the presence of competing Zn (I)-binding chelator (1,10-Phen). In the titration experiment, a fixed Zn\textsubscript{7}MT-2 (5 µM) concentration was used with increasing 1,10-Phen concentration from 20 to 800 µM, supplemented with 1 mM of DTT. In order to determine the binding constant, the free Zn (I) ion and the fractional occupancy of the Zn\textsubscript{7}MT-2 was calculated for each 1,10-Phen concentration from the MS spectra, and fitted with the free Zn(I) concentration in solution.

**Figure 19.** ESI-MS of Zn\textsubscript{7} MT-2 to 1, 10-phenanthroline at different concentrations (20M, 50M, 100M, 200M, 300M, 400M, 500M, 600M, 700M, 800M) *and ** refers Holo.MT and Apo-MT. Numbers refers ion charge state of respective.
Figure 20. Holo-MT to Apo-MT by increasing concentration of 1,10-phenanthroline (20µM, 50µM, 100µM, 200µM, 300µM, 400µM, 500µM, 600µM, 700µM, 800µM)

4.4. Metallothionein binding with other metals

To characterize metal ion binding of the peptides, the experiments were attempted in ammonium acetate buffer solution was used beside metals ion such as (Ag\(^{+}\), Au\(^{3+}\), Co\(^{2+}\), Cu\(^{2+}\), Hg\(^{2+}\), and Zn\(^{2+}\)) by using three molar ratios, such as 1:1, 1:10, 1:50. However, metal-free (apo) MT protein reoxidation occurred rapidly in the absence of zinc. Therefore, reduced with DTT, an ESI-MS compatible reducing agent. However, during prolonged storage times the protein were found to reoxidize even in the presence of DTT. However, as DTT can also chelate zinc ions, the use of excessive DTT was avoided in further experiments.
Figure 21. ESI FT-ICR mass spectra of (apo)MT-2 protein with 10-fold molar excess of a) Au$^{3+}$, b) Ag$^+$, c) Cu$^{2+}$, d) Co$^{2+}$, and e) Hg$^{2+}$ ions. The protein concentration was 6 μM in each. Numbers refers to ion charge state whereas * shows one, two, three, four, five, six, and seven times binding of respective.

A mass spectrum measured for (apo) MT-2 in the existence of 10-fold molar excess of metals ion such as (Ag$^+$, Au$^{3+}$, Co$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, and Zn$^{2+}$) ions showed a marked change in the CSD (4+ to 5+) as compared to the apo-protein and after metal binding in figure 21. The specificity of MT-2 to bind with different metals was assessed by using a variety of other alkali and transition metals. Small amount of the (apo) protein was observed in some samples. Metallothionein binding with other metals reveals that MT-2 was able to bind Ag$^+$, and Co$^{2+}$ but with low affinity. MT binds with both metals specifically such as Ag$^+$ shows one, two, four, five, six and seven times binding whereas Co$^{2+}$ reveals one, two, three, five, six and seven times binding. In addition, surprisingly, Hg$^{2+}$ ions were seen to bind with the protein from four to seven times. Only gold Au$^{3+}$ and Cooper Cu$^{2+}$ were able to bind with the MT-2 protein figure 21. The binding of the gold and copper ion to