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Non-coordinative metal selectivity bias in human metallothioneins metal-thiolate clusters

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Abstract

Mammalian metallothioneins (MT-1 through MT-4) are a class of metal binding proteins containing two metal-thiolate clusters formed through the preferential coordination of d¹⁰ metals, Cu(I) and Zn(II), by 20 conserved cysteine residues located in two protein domains. MT metalation (homometallic or heterometallic Zn(II)/Cu(I) species) appears to be isoform specific and controlling zinc and copper concentrations to perform specific and distinct biological functions. Structural and functional relationships, and *in vivo* metalation studies, identified evolutionary features defining the metal-selectivity nature for MTs. Metallothionein-3 (MT-3) has been shown to possess the most pronounced Cu-thionein character forming Cu(I)-containing species more favorably than metallothionein-2 (MT-2), which possesses the strongest Zn-thionein character. In this work, we identify isoform-specific determinants which control metal binding selectivity bias in different MTs isoforms. By studying the reactivity of Zn₇MT-2, Zn₇MT-3 and Zn₇MT-3 mutants towards Cu(II) to form Cu(I)₄Zn₄MTs, we have identified isoform-specific key non-coordinating residues governing folding/outer sphere control of metal selectivity bias in MTs metal clusters. By mutating selected residues and motifs in MT-3 to the corresponding MT-2 amino acids, we dissected key roles in modulating cluster dynamic and metal exchange rates, in increasing the Cu(I)-affinity in MT-3 N-terminal β-domain and/or modulating the higher stability of the Zn(II)-thiolate cluster in MT-2 β-domain. We thus engineered MT-3 variants in which the copper-thionein character is converted into a zinc-thionein. These results provide new insights into the molecular determinants governing metal selectivity in metal-thiolate clusters.

Graphical Abstract

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

INTRODUCTION

Metallothioneins (MTs) are a large superfamily of cysteine-rich metal-binding proteins and peptides characterized by the presence of single or multiple metal-thiolate clusters in their three-dimensional structures¹. Metal clusters in MTs are usually formed through the preferential coordination of d^{10} metal ions (e.g. Zn(II), Cd(II), and Cu(I)), by an array of closely spaced cysteine thiolate ligands — and in few cases, histidine — present in their primary sequence. The pattern of cysteine distribution in the MT sequence, the number of domains, and the number of metals in each cluster varies among the different MT families as a function of the taxonomy of the species from which MTs are isolated¹.

Mammalian MTs possess a dumbbell-like topology with two separated domains linked by a flexible hinge region². Each domain encompasses a metal-thiolate cluster. When bound to divalent metal ions, a $M(II)_3CysS_9$ cluster is present in the N-terminal β -domain and a $M(II)_4CysS_{11}$ cluster in the C-terminal α -domain ($M(II) = Cd(II)$ or $Zn(II)$), with all the 20 conserved cysteines involved in metal coordination^{3–7}. All metals are coordinated in a tetrahedral geometry by μ_2 -bridging and terminal cysteine thiolates. Contrarily, Cu(I) ions are coordinated in digonal or trigonal coordination, resulting in different cluster structures and as well different folding of the polypeptide backbone around the metal-thiolate cores within each domain^{8, 9}.

MT metalation state appears to be isoform specific and matching the zinc or copper buffering concentrations to perform tissue specific physiological functions. In mammals, MT-1 and MT-2 isoforms are ubiquitously expressed and play multiple physiological functions related to zinc homeostasis, metal buffering as well as metal detoxification — e.g. upon cadmium exposure². Under normal physiological conditions, MT-1 and MT-2 are isolated as homometallic, fully (or partially) metalated zinc-containing species^{2, 10–12}. Contrarily, native MT-3, as isolated from human¹³ and bovine¹⁴ brains, contains four Cu(I) and three to four Zn(II) ions within two homometallic clusters—a $Cu(I)_4$ -thiolate cluster in its N-terminal β -domain and a Zn_{3-4} -thiolate cluster in its C-terminal α -domain^{15, 16}. The isolated $Cu(I)_4Zn_4$ MT-3 is a monomeric protein that is stable in air^{14, 15}. This is in agreement with the unique biological and chemical functions of MT-3 in the central nervous system (CNS) in modulating neuronal zinc and copper metabolism. MT-3 possesses biological functions not shared by other MTs, including neuronal growth inhibitory activity and protection from amyloid- β toxicity^{17, 18}. A protective role from Cu toxicity—Cu(I)

intracellularly and Cu(II) extracellularly, for both intracellular and secreted extracellular Zn₇MT-3 has also been suggested on the basis of investigations of its reactivity toward Cu(I) and Cu(II) ions. Zn₇MT-3 can efficiently reduce Cu(II) to Cu(I) and bind it with high affinity, forming air-stable Cu(I)₄Zn₄MT-3 species. In this reaction, a Cu(I)₄-thiolate cluster is formed cooperatively with concomitant release of three Zn(II) ions¹⁹. With similar reactivity, Zn₇MT-3 efficiently controls aberrant copper-protein interactions, which underlie a number of neurodegenerative disorders. Zn₇MT-3, through Cu(II) removal from amyloid- β , α -Synuclein and prion protein via metal swap reactions, leads to the formation of air-stable Cu(I)₄Zn₄MT-3 species, efficiently preventing the deleterious redox activity and toxicity of these protein and peptide metal complexes^{20–23}.

In vitro formation of MTs species with higher Cu(I) stoichiometry can be obtained via apothionein metalation or Cu(I) titration in Cu(I)₄Zn(II)₄MTs under reducing conditions — e.g. Cu(I)₈, Cu(I)₁₀, up to Cu(I)₁₂ in solution, with evidence up to Cu(I)₂₀ demonstrate only in gas phase^{24–27}. However, MT Cu(I) metalation beyond the Cu(I)₄ cluster, as observed in native Cu(I)₄Zn₄MT-3, appears to be limited exclusively to pathological situations resulting from Cu(I) overload — e.g. MT-1/–2 in Wilson's disease liver^{9, 28}. The preference for discrete species with higher distinct Cu(I)-binding stoichiometries arise from cooperativity in the formation of Cu(I)_{4/6}-thiolate clusters¹.

A fundamental question about MTs metal-thiolate clusters is what determinants control metal binding selectivity bias in different isoforms. Based on the structural/functional relationships and *in vivo* metalation studies of different mammalian MT isoforms expressed in *E. coli*, a classification of MTs as zinc-thioneins or copper-thioneins has been proposed by pioneering work of Atrian, Capdevila and coworkers, in the efforts to identify evolutionary features defining metal-selectivity nature for MTs^{26, 29}. Among the 4 mammalian isoforms, MT-3 was shown to possess the most pronounced Cu-thionein character with propensity to form Cu(I)-containing species more favorably than MT-2, which in turn shows the strongest Zn-thionein character. The remaining MT-1 and MT-4 possess intermediate characters with the former shifted towards being a zinc-thionein and MT-4 more towards a copper-thionein^{30–32}. The metal selectivity character underlies fundamental roles played by MT-3 in copper homeostasis and controlling aberrant copper-protein interactions, and in zinc buffering and homeostasis by MT-2^{2, 23}. However, the structural, sequence, and reactivity determinants in MT sequences responsible for their respective selectivity bias in their metal-thiolate clusters has so far remained elusive.

In this study, we reveal the role of key non-coordinating residues in folding and outer sphere control of metal selectivity bias in metallothionein clusters and provide new information on the key sequence feature which control higher Cu(I) selectivity in MT-3. We investigated reactivity of MTs towards Cu(II), the kinetics of metal exchange and the thermodynamic stability of metal-thiolate clusters in MT-3, MT-2 and in MT-3 mutants in which key residues have been mutated to the corresponding ones present in MT-2. Despite the role of some non-coordinating residues in controlling relative stability and reactivity in Cd(II)/Zn(II) clusters in mammalian MTs has been characterized, differences in cluster nuclearity, cluster structure and polypeptide folding in Zn(II)/Cd(II) MTs compared to Cu(I)-containing MTs prevented molecular understanding on how the Cu(I) nature bias is obtained in MTs. In

this study, we reveal specific roles played by non-coordinative residues in modulating metal exchange rates towards Cu(I), in increasing the Cu(I)-affinity in MT-3 N-terminal β -domain and/or modulating the higher stability and affinity towards Zn(II) in MT-2 β -domain. Through selective mutations, we have engineered MT-3 variants in which the copper-thionein is converted into a zinc-thionein. In MT-2 and MT-3, the array of 20 cysteine coordinating residues in the metal-clusters is fully conserved (Figure 1) and results in equal cluster structures. The identified non-coordinating residues play critical roles in MT-3/MT-2 proteins and confer a copper- or zinc-thionein character to match buffered metal pools in cells and the distinct physiological roles played by these different isoforms.

RESULTS AND DISCUSSION

Reaction of Zn₇-MTs with Cu²⁺ and formation of Cu(I)₄Zn(II)₄MTs

Previous work demonstrated that Zn₇metallothionein-3 can efficiently reduce Cu(II) ions to Cu(I) and bind Cu(I) in metal-thiolate clusters within its two domains¹⁹. The reaction of Zn₇metallothionein-3 with up to 4 Cu(II) equivalents leads to the cooperative formation of Cu(I)₄Zn(II)₄MT-3 species in which an oxygen-stable Cu(I)₄-thiolate cluster is present in its N-terminal β -domain and 3 Zn(II) ions are released from this domain together with formation of two disulfide bonds³³. The same reactivity has not been characterized in detail for MT-2. We have therefore performed a comparative characterization of the products of the reactions between Zn₇MT-3/Zn₇MT-2 and Cu(II) by employing a combination of spectroscopic and spectrometric techniques. The absorption spectra of mammalian Zn₇MTs, including Zn₇MT-2 and Zn₇MT-3, are characterized by the absence of polypeptide derived absorption above 240 nm due to the lack of aromatic or histidine residues, and by a metal-induced shoulder around 235 nm, originating from CysS–Zn(II) ligand-to-metal (LMCT) transitions (Figure 2). Upon reaction with 4 Cu²⁺ equivalents, the absorption spectrum shows a decrease in absorbance below 230 nm corresponding to decreased CysS–Zn(II) LMCT contributions arising from Zn(II) release, and an increase in absorbance above 230 nm which results in a pronounced shoulder centered at 260 nm, arising from CysS–Cu(I) LMCT and pinpointing Cu(II) reduction and Cu(I) binding (Figure 2). These spectroscopic features resulting in an isosbestic point at 230 nm are diagnostic of Cu(II) reduction and Cu(I) binding to thiolates, in agreement with the formation of Cu(I)₄Zn(II)₄MT-3. Characterization of the reaction of Zn₇MT-2 with 4 Cu(II) equivalents results in almost identical spectroscopic features corresponding to the formation of similar Cu(I)/Zn(II)MT-2 species. We utilized Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine metal-to-protein stoichiometry and confirmed the formation of Cu(I)₄Zn₄MT-2 species (Supplementary Table 1). We confirmed the presence of a Cu(I)₄-thiolate cluster with similar structure by analyzing its low-temperature 77 K luminescence spectrum upon comparison to the spectrum of Cu(I)₄Zn₄MT-3. Cu(I)₄Zn₄MT-2 shows an emission spectrum characterized by a high energy band at ~ 430 nm and a low energy band at ~ 575 nm with lifetimes of $\tau_{425} = 46 \mu\text{s}$ and $\tau_{575} = 131 \mu\text{s}$ (Figure 3), respectively. These features are identical to the ones observed in Cu(I)₄Zn₄MT-3³³. The presence of these two emissive bands, with cluster-centered and charge-transfer origins, respectively, together with their characteristic lifetimes, are diagnostic of the presence a Cu(I)₄ cluster with Cu-Cu distances <2.8 Å in the Cu(I)₄Zn₄MT-2 structure. Based on this spectroscopic characterization and in light of the

demonstrated localization of the Cu(I)₄-thiolate cluster in Cu(I)₄Zn₄MT-3 β -domain, together with the higher affinity of MTs β -domains for Cu(I) over the α -domains⁸, we conclude that similar Cu(I)₄ and Zn₄-clusters in the β - and α - domains are formed in Cu(I)₄Zn₄MT-2 generated upon reaction of Zn₇MT-2 with Cu(II) ions. Thus, the copper-over zinc- thionein character does not stem from differences in metal cluster nuclearity generated in each of the two isoforms.

Stability of Cu(I)/Zn(II)MT-2 and Cu(I)/Zn(II)MT-3 towards oxidation

Cu(I)₄Zn₄MT-3 is characterized by a unique stability of its Cu(I)₄ cluster towards oxidation in the presence of molecular oxygen. To investigate whether differences in redox stability of the metal clusters underlie the metal character in each isoform, we characterized the stability towards oxidation in Cu(I)/Zn(II)MTs. Zn₇MT-2 and Zn₇MT-3 samples were titrated with 0, 4, or 8 Cu²⁺ equivalents, incubated for 1 h or 24 h at 37 °C, and subjected to size exclusion chromatography (SEC) analysis. We monitored the sample absorption at 220 nm and 260 nm where contributions from CysS-Zn(II) and CysS-Cu(I) LMCT, respectively, allow to monitor Zn(II) and Cu(I) release over time consequent to thiolate oxidation to disulfides. As expected, both Zn₇MT-2 and Zn₇MT-3 were air-stable as indicated by identical peak areas in the SEC elution profiles upon 24 h incubation in air (Figure 4). Similarly, the reaction with 4 Cu²⁺ equivalents generating Cu(I)₄Zn₄MTs resulted in the formation of air-stable proteins with identical peak integrals after 24 h incubation (Figure 4). A shift in retention volume was observed in these samples for both isoforms, indicating a decrease in the Stokes' radius upon Cu⁺ binding as previously observed for Cu(I)₄Zn₄MT-3¹⁹. Contrarily, reaction of both proteins with 8 Cu²⁺ equivalents — which results into Cu(I) binding to the α -domain— resulted in a major shift in their retention concomitant to > 75% decrease in absorption upon 24 h incubation of the products of the reaction in air. This indicated an extensive thiolate oxidation and concomitant metal-release (Figure 4 and Supplementary Table 2). In Cu(I)₄Zn₄MT-3, an air-stable Cu(I)₄-thiolate cluster was demonstrated to form cooperatively in the N-terminal domain. Thus, a similar air-stable Cu(I)₄ cluster is present in the N-terminal domain of Cu(I)₄Zn₄MT-2.

Cu(I)-binding affinity in Cu(I)₄-thiolate clusters of Cu(I)₄Zn₄MT-2 and Cu(I)₄Zn₄MT-3

Cu(I)-binding affinity in MT-thiolate clusters has been reported to be in the order of $K_d \cong 1 \times 10^{-12}$ – 1×10^{-19} M, depending on the method used for the determination and the metal species/isoform investigated^{24, 34, 35}. Based on the Atrian and Capdevila classification^{26, 29}, despite both MT-2 and MT-3 preferentially bind Cu(I) over Zn(II), the difference in affinity for Cu(I) over Zn(II) ought to be larger for MT-3 than MT-2. Hence, *in vivo* Cu(I)-binding to MT-3 is more favorable when compared to other human MT isoforms. The higher Cu(I)-binding character of MT-3 compared to MT-2 is assigned to its β -domain²⁶. However, no relative quantitative determination of specific copper affinities of the Cu(I)₄-thiolate cluster for mammalian Cu(I)₄Zn₄MT-2 and Cu(I)₄Zn₄MT-3 is available.

To verify the relative affinity preference for Cu(I) towards MT-3, a preliminary competition experiment was conducted by reacting an equimolar mixture of Zn₇MT-2 (5 μ M) and Zn₇MT-3 (5 μ M) with 20 μ M Cu²⁺ for 1 h followed by protein separation by SEC (Supplementary Figure 1). MT-2 and MT-3 elution peaks are partially overlapping (MT-3

elution volume $\cong$ 13 ml, MT-2 $\cong$ 13.8 ml). Nevertheless, samples corresponding to the center of each peak (12.92–13.12 ml for MT-3 and 13.72–13.92 ml for MT-2) were analyzed for metal content using ICP-MS. The results revealed that the peak corresponding to MT-3 was enriched with more Cu(I) than the MT-2 peak, suggesting that MT-3 possesses a higher Cu(I) binding affinity in its β -domain Cu(I)₄-thiolate cluster.

We therefore quantitatively investigated the Cu(I) affinities of Cu(I)₄Zn₄MT-2 and Cu(I)₄Zn₄MT-3 by performing competition reactions with Cu(I) affinity probes to determine average Cu(I) dissociation constants. Initial experiments performed using Bicinchoninate (Bca), a bidentate ligand with strong affinity for Cu⁺ which forms a [Cu(I)Bca₂]³⁻ complex with a formation constant of $\beta_2 = 10^{17.2} \text{ M}^{-2}$ ³⁶, revealed that the overall average Cu(I) affinity in MTs was very high ($< 10^{-17} \text{ M}$) and that Bca could efficiently compete with MT sites only at mM concentrations.

Therefore in subsequent experiments, we have utilized bathocuproine disulfonate (Bcs) as a copper affinity probe³⁶. Bcs is a bidentate ligand with high affinity for Cu⁺ that generates a [Cu(I)Bcs₂]³⁻ complex with a formation constant of $\beta_2 = 10^{19.8} \text{ M}^{-2}$. The [Cu(I)Bcs₂]³⁻ complex has a maximum absorption at 483 nm with $\epsilon_{483} = 13000 \text{ M}^{-1} \text{ cm}^{-1}$, allowing for quantification of [Cu(I)Bcs₂]³⁻ at equilibrium and determination of a Cu(I) dissociation constant (K_d) for Cu(I) binding proteins³⁶ (see Material and Methods). Oxygen-free 10- μM Cu(I)₄Zn₄MT-2 or Cu(I)₄Zn₄MT-3 samples (pH 7.4) were reacted in anaerobic atmosphere with 1 mM Bcs at 25 °C, and the amount of Cu(I) bound and metal-free ligand and protein species determined after 15 h by absorption spectroscopy. The concentration of Bcs used was determined from preliminary experiments which showed that 1 mM Bcs allows for an equilibrium competition environment between MTs (see also below) and Bcs, where 30–60% of Cu(I) is chelated from the Cu(I)₄Zn₄MT proteins. Calculation of the average Cu(I) dissociation constants for Cu(I) sites in Cu(I)₄Zn₄MT-2 and Cu(I)₄Zn₄MT-3 revealed that MT-3 possesses an approximately ten-times higher binding affinity for Cu(I) with an average dissociation constant (K_d) of $5.64 \times 10^{-20} \text{ M}$ compared to MT-2, for which the K_d is $4.32 \times 10^{-19} \text{ M}$ (Table 1). These results reveal that the Cu(I)-thionein nature correlates with the higher affinity for Cu(I) in the Cu(I)₄-thiolate cluster in the β -domain of heterometallic Cu(I)₄Zn₄MT species. MT-3 affinity ranges in the expected Cu(I) buffering capability to prevent the presence of equilibrium “free” copper in biological milieu³⁷, and to control labile copper pools in signaling and/or upon synaptic release^{35, 38}.

Comparative Cu(I)/Zn(II) exchange kinetics in MT-2 and MT-3

In light of the formation of similar Cu(I)₄Zn₄MT species but with differences in the thermodynamic stability of its Cu(I)₄-thiolate cluster, we aimed at investigating whether differences in metal exchange reaction kinetics also contribute to the Cu(I)-thionein nature of MT-3 and the Zn-thionein character of MT-2. It is expected that, in biological environment, kinetic bias can contribute in determining MTs metal content. In light of the different energies of CysS-M LMCT for Zn(II) and Cu(I) as described above, we performed stopped-flow spectroscopic measurements to monitor the kinetics of Zn²⁺ release and Cu⁺ binding to form the Cu(I)₄ cluster in MTs. Our stopped-flow spectroscopy setup allows spectroscopic observation of reactions upon mixing of the two reactants, with a dead time of

approximately 2–3 ms. We have utilized this setup to follow the reaction of 2.5 μM Zn₇MT-3 with 4 Cu^{2+} equivalents at 25 °C. Zn²⁺ release was monitored at 220 nm while Cu⁺ binding was monitored at 260 nm, and the results reported as a fraction of relative LMCT intensity over the total change upon reaction completion thereby representing the Zn²⁺ and Cu⁺ bound to MTs at a given time (Figure 5). The exchange reaction was complete (>95%) in 75 s for MT-3 while it required 250 s for MT-2. Within 25 s of reaction, 65% of Zn²⁺ is released and 75% of Cu⁺ is bound to MT-3, showing that Cu(I) binding is kinetically concomitant to Zn(II) release. Performing the same experiment with Zn₇MT-2, after 25 s, only 35% of the Cu⁺ is bound. Apparent initial rates under the conditions used were determined from exponential fitting for the Cu(I) binding traces revealing that MT-3 rates ($0.30 [\mu\text{M Cu}]/[\mu\text{M MT}] \text{ s}^{-1}$) were five times faster than MT-2 ($0.06 [\mu\text{M Cu}]/[\mu\text{M MT}] \text{ s}^{-1}$, see below). Thus, in MT-3, Cu(II) reduction and Cu(I)/Zn(II) exchange in its β -domain is significantly faster than in MT-2 (Figure 6).

Selection and characterization of Cu(I)₄Zn₄MT-3 mutants

The obtained results indicated that MT-3 possesses a pronounced Cu(I) selectivity bias over zinc in its N-terminal β -domain which stems from unique features intrinsic to its amino acid sequence. In all mammalian isoforms of MTs, the metal-coordinating cysteine residues are fully conserved, indicating that selectivity bias must lay in their non-coordinating residues².

To identify specific roles of key non-coordinating residues in determining copper selectivity bias in MT-3, we have generated and characterized a series of point and combination mutants in which key residues exclusively present and conserved in MT-3 sequences and/or important for its biological activity (but absent in MT-2 sequences) (Supplementary Figure 2) were mutated to the corresponding MT-2 amino acids (Figure 7). Particular focus was placed on β -domain residues where the Cu(I)₄-thiolate cluster is present in the Cu(I)₄Zn₄MT-3 species. MT-3 β -domain sequences are characterized by a conserved Thr insert in position 5 followed by a Cys-Pro-Cys-Pro motif which is absent in MT-2. Both features are necessary for the neuronal growth inhibitory activity of MT-3 that is not shared by other MT isoforms^{39, 40}. In addition to those, a Glu at position 23 and Gly at position 24 are highly conserved in all mammalian MT-3s but are divergent in MT-2 sequences (Supplementary Figure 2). Despite this sequence feature is not 100% conserved in MT-3, it was selected in our investigation because mutations of Glu23 to a corresponding Lys residue, while not significantly altering the structure of MT-3, eliminates its neuronal growth activity.⁴¹ In addition to these key features in the β -domain, another key difference between MT-3 and MT-2 sequences is the presence of an acidic six-amino acid insert in its α -domain (in human MT-3 corresponding to Glu-Ala-Ala-Glu-Ala-Glu in position 55–60 of MT-3 α -domain). The sequence of this insert is not strictly conserved in all MT-3 sequences but its presence and its acidic nature are features preserved in MT-3s. Deletion of this insert results in a remarkable reduction in MT-3 biological activity. Moreover, the sequence was found to be responsible in lowering the stability of the α -domain metal clusters in MT-3, probably causing the polypeptide surface and cluster core to be more solvent accessible⁴².

MT-3 single point and combination mutants were generated, expressed as Cd(II) bound forms, purified, and Zn₇MTs forms generated via metal reconstitution as described in

Material and Methods. For all mutants, the protein purity was confirmed by SDS-PAGE upon monobromobimane modification, where a single band corresponding to MTs was observed (Supplementary Figure 3), and zinc-to-protein ratios of 7.0 ± 0.6 and CysSH-to-protein ratios of 20 ± 3 were obtained (Supplementary Table 3). The reactions of all these Zn₇MT-3 mutants with 4 Cu²⁺ eq. was performed as described for MT-2/MT-3 and the metal binding stoichiometry for all the products determined by ICP-MS and protein quantification. For all Zn₇MT mutants, a ratio of 4.0 ± 0.8 Zn(II) eq/MT and 4.0 ± 0.6 Cu(I) eq/MT was determined, consistent with the generation of Cu(I)₄Zn₄MT species (Supplementary Table 1). The presence of similar Cu(I)₄-thiolate clusters was confirmed by measuring the low-temperature luminescence spectra and emission lifetimes for all the Cu(I)₄Zn₄mutants. The presence two emissive bands centered at 425 nm and 575 nm (Figure 8) with lifetimes of $\tau_{425} \sim 47 \mu\text{s}$ and $\tau_{575} \sim 130 \mu\text{s}$, respectively (fitted using a single exponential decay function, Supplementary Table 5), revealed that all Cu(I)₄Zn₄MT species generated feature a Cu(I)₄ cluster with very similar structural features and Cu-Cu distances as observed in Cu(I)₄Zn₄MT-2 and Cu(I)₄Zn₄MT-3. Thus, mutations of all these conserved non-coordinating residues in MT-3 does not affect the formation of Cu(I)₄Zn₄MTs species possessing a Cu(I)₄-thiolate cluster in the N-terminal β -domain and a Zn(II)₄-thiolate cluster in their C-terminal α -domain. Thus, this platform of Zn₇MT-3/Cu(I)₄Zn₄MT-3 mutants allowed us to investigate specific roles played by non-coordinating residues in controlling the kinetics of Cu(I)/Zn(II) exchange and modulating the relative Cu(I) and Zn(II) affinity in MT-3 and MT-2 metal-thiolate clusters.

Kinetics of Cu(I)-binding in MT-3 mutants

To study the effect of mutation of MT-3 residues to corresponding MT-2 residues on the kinetics of Cu(II) reduction and exchange with Zn(II), the reaction of Zn₇MT-3 mutants with Cu(II) was studied using stopped-flow absorption spectroscopy. The reaction of 2.5 μM Zn₇MTs with 4 Cu²⁺ equivalents at 25 °C was followed at 260 nm to quantify the fraction of bound Cu(I) as a function of time (Figure 9). Apparent initial rates under the conditions used were determined from the derivative of an exponential fitting for the Cu(I) binding traces and reported in Supplementary Table 4. Deletion of Thr5 or mutation of Glu23 and Gly24 do not show to have any effect on the kinetics of Cu(I) binding to form the Cu(I)₄-thiolate cluster as the observed initial rates for T5 MT-3, E23K MT-3 and E23KG24E MT-3 were similar to the one of wild-type Zn₇MT-3. Contrarily, mutants in the Cys6-Pro7-Cys8-Pro9 motif in the β -domain and the acidic insert in the α -domain both significantly affected the kinetic of Cu(II) reduction/Cu(I) binding. The observed initial rates for P7SP9A MT-3 ($0.11 \pm 0.02 [\mu\text{M Cu}]/[\mu\text{M MT}] * \text{s}^{-1}$) and 55–60 MT-3 ($0.14 \pm 0.02 [\mu\text{M Cu}]/[\mu\text{M MT}] * \text{s}^{-1}$) were intermediate between the one of w₇MT-3 (which showed the fastest rate: $0.30 \pm 0.04 [\mu\text{M Cu}]/[\mu\text{M MT}] * \text{s}^{-1}$) and the one of MT-2 (slowest rate: $0.06 \pm 0.01 [\mu\text{M Cu}]/[\mu\text{M MT}] * \text{s}^{-1}$). Combination mutants in which Thr5 is deleted and Glu23 is mutated together with the P₆CPC₉ motif (T5-P7SP9A MT-3 and T5-P7SP9A-E23K MT-3) caused no major decrease in the rates when compared with P7SP9A MT-3. On the other hand, combined mutation of both the Cys6-Pro7-Cys8-Pro9 motif together with the acidic insert, deletion of Thr in position 5, and mutation of Glu 23 resulted in a further reduction in the reaction rates resulting in an engineered protein which reacted with the same initial rate as MT-2.

Overall, the reaction rates followed the order:

These results reveal that presence of the prolines in the Thr5-Cys6-Pro7-Cys8-Pro9 motif are critical, together with the presence of the acidic insert in the α -domain, in modulating the faster Cu(I)/Zn(II) exchange reactions observed in MT-3 with additional minor roles played by Thr5 and Glu23 residues. Thus, the Cu(I)-thionein nature of MT-3 stems partially from modulation of the rates of Cu(I) binding and Zn(II) release mediated by critical non-coordinating residues which are present in both the β - and α -domains. These results also reveal important inter-domain interactions in tuning the metal exchange properties in MT-3 β -domain.

Previous work by Vasak and coworkers on Cd₇MT-3 demonstrated that proline mutations in the Cys6-Pro7-Cys8-Pro9 motif of MT-3 are responsible for altering the unique cluster dynamic in the Cd(II)₃-thiolate cluster present in MT-3 β -domain^{40, 43}. 2D ¹H-¹⁵N Heteronuclear Single Quantum Coherence (HSQC) spectroscopy confirmed that the N-terminal 12 residues are more flexible than other regions of the protein in M(II)₇MT-3, implying that the Thr5-Cys6-Pro7-Cys8-Pro9 sequence at the N-terminus contribute to the dynamics of the β -domain of MT-3, which prevented the determination of the structure of the β -domain by NMR^{44, 45}. Thus, the increased dynamics of the metal-thiolate cluster in MT-3 β -domain compared to MT-2, when bound to divalent metal ions, results in an increased solvent accessibility of the metal-bound thiolates which cause faster Cu(II) reduction, Zn(II) release, and Cu(I) binding rates, giving rise to the faster Cu(I)/Zn(II) exchange rates in MT-3 observed in this work. In addition to the Cys6-Pro7-Cys8-Pro9 motif, another major role is played by the acidic insert in the α -domain. Inter-domain interactions have been demonstrated to be critical in modulating the destabilization of the β -domain of MT-3 and the amino acid insert plays an important role in this process. Deletion of the E₅₅AAEAE₆₀ insert reduces the reaction rates of Zn₇MT-3 with thiophilic reactants such as 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and S-nitrosocysteine (SNOC), and on the other hand increases the stability of the metal-thiolate cluster in the β -domain^{42, 46}. Despite only the structure of MT-3 Cd₄ α -domain could be determined by NMR, the simulated structure of Zn₇MT-3 and its 55-60 mutant revealed that the EAAEAE insert affects the interaction between the interdomain linker region and the α -domain of MT-3⁴⁷. In wMT-3, these interactions are speculated to have a critical influence on the structure of the β -domain in which a different hydrogen-bond network generates a β -domain Zn(II)₃-thiolate cluster that is more solvent exposed and dynamic when compared to the one in Zn₇MT-2⁴⁷. Thus, the Cys6-Pro7-Cys8-Pro9 motif and the acidic insert are likely critical in determining the faster Cu(II) reduction rates by generating more accessible thiolates in MT-3 β -domain and subsequently causing significantly faster Cu(I)/Zn(II) exchange rates because of the more dynamic nature of the domain when bound to Zn(II), which is modulated through inter-domain interactions. It is worth noting that Thr5 plays only a minor role in controlling the exchange kinetic rates. Therefore, we investigated whether this and other

residues are critical in generating a bias in the polypeptide folding/outer sphere effects that results in a change of the relative stability of Cu(I)- and Zn(II)-thiolate clusters in the β -domain of MT-2 and MT-3.

Cu(I)-binding affinity in $\text{Cu(I)}_4\text{Zn(II)}_4$ MT-3 mutants

While the faster exchange rate in MT-3 can be ascribed to the increased dynamics in MT-3 β -domain which underlies a faster Zn(II) release and faster metal exchange, the generated Cu(I)_4 cluster in MT-3 is on the other hand more stable than the corresponding cluster in MT-2 suggesting that these dynamic processes in MT-3 are not maintained when Cu(I) is bound. Thus, the polypeptides backbone fold in $\text{Cu}_4(\text{I})\text{Zn}(\text{II})_4\text{MT-3}$ must give rise to an overall structure that better stabilize the Cu(I)_4 core in MT-3.

To gain further insight into the role of key residues in determining the Cu(I)-thionein nature of MT-3, we have determined the average Cu(I) binding constant in all generated mutants to determine which non-coordinating residues control the higher β -domain Cu(I)-binding affinity in $\text{Cu(I)}_4\text{Zn(II)}_4\text{MT-3}$.

The dissociation constants (K_d) for all mutants were determined using the Bcs methods as described for $w\text{Cu(I)}_4\text{Zn(II)}_4\text{MT-2}$ and $w\text{Cu(I)}_4\text{Zn(II)}_4\text{MT-3}$.

The results are reported in Table 1. The obtained results revealed that the Cu(I)-binding affinity for MTs follows the order:

All mutations in the MT-3 sequence to the corresponding amino acid present in MT-2 caused an increase in the determined average Cu(I) dissociation constant. Mutations of Thr5, the Cys6-Pro7-Cys8-Pro9 motif, Glu23 and the acidic insert all showed a major effect in reducing the Cu(I)-binding affinity with all these mutants possessing an average affinity that is approximately 2.5 time smaller that of MT-3. The role of all the amino acids in determining the Cu(I)-affinity in MT-3 are additive as combination mutants show progressively increasing K_d s with the one determined for T5-P7SP9A-E23KG24E-55-60 MT-3 ($K_d = 2.99 \pm 0.53 \times 10^{-19}$ M) approaching the K_d value of MT-2 ($K_d = 4.32 \pm 0.8 \times 10^{-19}$ M).

These experiments support distinct roles for specific MT-3 amino acids in modulation of metal exchange rates and/or stability of the Cu(I)_4 -thiolate cluster. No structure of $\text{Cu(I)}_4\text{Zn}_4\text{MTs}$ is currently available and only models of the structure of the Cu(I)_4 -thiolate core have been proposed⁹. However, an investigation of isolated β -domain of MT-1 reconstituted with 3 Zn(II) or 4 Cu(I) provided clear evidence of stable polypeptide folds that are largely different in the two species, caused by different clusters structures and metal coordination geometries. It is expected that the polypeptide fold in the β -domain of $\text{Cu(I)}_4\text{Zn}_4\text{MT-2}$ and $\text{Cu(I)}_4\text{Zn}_4\text{MT-3}$ ought to be different compared to the corresponding $\text{Zn}_7\text{MT-2/-3}$ species. It can be postulated that the higher stability for the Cu(I)_4 -cluster has

to arise from the presence of unique residues which result in a polypeptide fold/outer sphere effects that allow a larger stabilization energy for the Cu(I)₄-thiolate core in MT-3.

While only single mutations of the Cys6-Pro7-Cys8-Pro9 motif and acidic insert significantly affect reaction kinetics, also a single mutation of Thr5 and Glu23 had major effect in reducing the Cu(I) binding affinity. It has been speculated that Thr5 is essential in the formation of a distinct hydrogen-bonding network in MT-3 as its deletion affect both the Cu(I)₄-thiolate cluster affinity determined in this study, as well as its neuronal growth inhibitory activity⁴⁸. In agreement, in a T5S mutant which maintain a similar hydrogen-bonding capability through its side-chain hydroxyl group, a growth inhibitory activity comparable to that of the wild-type is present⁴⁸. The presence of the unique Thr5-Cys6-Pro7-Cys8-Pro9 motif in MT-3 in which the two prolines impose constraints in their *phi* angles could therefore underlie the higher binding affinity of the MT-3 β -domain towards Cu(I). A characteristic conformation of the Thr5-Cys6-Pro7-Cys8-Pro9 sequence was calculated in the β -domain of Zn₇MT-3, where both Pro7 and Pro9 faced outwards with their five-member rings in a parallel configuration and Thr5 at the opposite side of the two Pro rings⁴⁷. In light of the different coordination of Cu(I) (digonal or trigonal) and Zn(II) (tetrahedral) in the clusters, and the expected different polypeptides fold and Cys connectivity in Cu(I)₄ and Zn(II)₃ clusters, we speculate that this Thr5-Cys6-Pro7-Cys8-Pro9 motif constrains the polypeptide fold to a configuration which better favors folding around Cu(I)₄ core and destabilize the Zn₃ cluster when compared to MT-2. We speculate that this, together with the hydrogen-bonding capacities that can be mediated by Glu23 and the acidic insert through inter-domain interactions, might be critical to increase stability of the Cu(I)₄-thiolate core in MT-3 β -domain. In agreement with this role, also mutations of Glu23 abolishes neuronal growth inhibitory activity in MT-3. Overall, both polypeptide fold and outer sphere effects (via modulation of thiolate nucleophilic through hydrogen-bonding) mediated by these residues appear responsible for the increased Cu(I)-nature of MT-3 β -domain.

Key unique, non-coordinating amino acids in MT-3 sequence specifically modulate the Cu(I)/Zn(II) reaction rates and/or the final overall higher affinity of the Cu(I) cluster in MT-3. However, their effect in affecting the domain Zn(II)-thionein character required to be investigated.

Zn(II)-binding affinity of Zn₇MT-2, Zn₇MT-3 and Zn₇MT-3 mutants

Our results show that mutations in key MT-3 positions to the corresponding MT-2 residues in the sequence cause a decrease in its β -domain affinity for Cu(I). However, it is not known whether and how these residues also play a role in modulating the relative Zn(II) affinity between MT-3 and MT-2. To address this question, average Zn(II) dissociation constants were determined for Zn₇MT-2, Zn₇MT-3, and all the Zn₇MT-3 mutants. Although there is no consensus, it appears that not all the 7 Zn(II) ions possess the same affinity in MTs, with the presence for 4 higher affinity sites ($K_d = 1.6 \cdot 10^{-12}$ M) and three weaker sites ($K_d = 3.5 \cdot 10^{-11}$ M, $1.1 \cdot 10^{-10}$ M, $2.0 \cdot 10^{-8}$ M, respectively)¹¹. The exact identification of these 3 weaker sites, likely located in the β domain, and their corresponding affinity, still remains elusive. However, recent work by Krezel and coworkers on MT-2 Zn(II) metalation elegantly

demonstrated that the α -domain Zn(II)₄-thiolate cluster is formed sequentially in the first stages, followed by the sequential formation of the β -domain Zn(II)₃-thiolate cluster, although both processes partially overlap. The study also revealed that the weakest Zn(II) ions associates with the β -domain overall indicating that the 3 weaker sites are located in the N-terminal β -domain 3 metal cluster⁴⁹. We proceeded in determining the overall average Zn(II) binding affinities using the chromogenic zinc chelator 4-(2-pyridylazo)resorcinol (PAR). PAR forms a stable 2:1 complex with Zn(II), $\text{ZnH}_x(\text{PAR})_2$, with a high molar absorption coefficient ($\epsilon_{492}=71\,500\text{ M}^{-1}\text{ cm}^{-1}$) allowing for the detection of low Zn(II) concentrations⁵⁰. 1.43- μM Zn₇MT samples (pH 7.4) were reacted with 170 μM PAR for 2.5 h (resulting in 30–50 % Zn chelation from MTs). The absorbance at 492 nm was then measured, and average Zn(II) dissociation constants calculated using the effective dissociation constant of $\text{ZnH}_x(\text{PAR})_2$ at pH 7.4 ($K_d^{\text{eff}} = 7.08 \times 10^{-13}\text{ M}^2$)⁵⁰. The experimental conditions utilized allowed for efficient competition resulting in Zn(II) removal primarily from the β -domain and therefore in determination of average Zn K_d constants that primarily reflect stability differences in the 3 metal-clusters. The results confirmed that MT-3 possess lower average affinity for Zn(II), with a K_d of $1.29 \pm 0.05 \times 10^{-10}\text{ M}$, compared to MT-2, with a K_d of $3.06 \pm 0.12 \times 10^{-11}\text{ M}$. The decreased affinity has been associated to the presence of zinc sites with decreased affinity in the β -domain when compared to MT-2.

The determined average Zn(II) dissociation constants for all the mutant Zn₇MTs (Table 2) indicated that the Zn(II)-binding affinity follow the order:

Zn₇-MT-3 ~ T5 MT-3 ~ 55–60 MT-3 ~ E23K MT-3 ~ E23KG24E MT-3 <<
P7SP9A MT-3 < T5-P7SP9A MT-3 ~ T5-P7SP9A-E23K MT-3 ~ T5-P7SP9A-
55–60 MT-3 ~ T5-P7SP9A-E23K- 55–60 MT-3 ~ T5-P7SP9A-E23KG24E-
55–60 MT-3 ~ **Zn₇MT-2**

The results reveal that Zn(II) affinity follow an opposite order than the affinity for Cu(I), with Zn₇MT-2 showing the highest affinity and MT-3 the lowest. Solely the Cys6-Pro7-Cys8-Pro9 motif, together with minor contribution of the threonine insert in position 5, appear critical in conferring the lower average Zn(II) affinity in MT-3. Mutation to the Pro7/Pro9 residues to the corresponding Ser and Ala residues present in MT-2, together with Thr5, resulted in a 5-fold increase in zinc affinity. As a result, the T5-P7SP9A MT-3 possess a dissociation constant (K_d) of $4.18 \pm 0.05 \times 10^{-11}\text{ M}$ —compared to K_d of $1.29 \pm 0.12 \times 10^{-10}$ in Zn₇MT-3— which is close to the one of Zn₇MT-2 (K_d = $3.06 \pm 0.12 \times 10^{-11}\text{ M}$). Mutation of other residues did not affect significantly the overall zinc binding affinity, including deletion of the acidic insert. Thus, favorable bias for Zn(II) binding in MT-2 over MT-3, conferring a Zn-thionein character, stems from relative solvent accessibility, decreased metal-exchange rates, and increased thermodynamic stability of zinc-thiolate cluster in its β -domain. These results are in agreement with previous work on MT-3 and a double mutant in which the 2 prolines were mutated, showing that the Cys6-Pro7-Cys8-Pro9 motif plays critical roles in modulating the dynamic properties of the N-terminal β -domain and the relative binding affinity towards Zn(II)/Cd(II). Thus, the Cys6-Pro7-Cys8-Pro9 motif in MT-3 is critical in decreasing the Zn-thionein nature in MT-3 β -domain.

It should be noted that physiologically, MTs can exist as dynamic proteins with different metal bound species depending on the state of the cells and the tissue of origin, with Zn₅, Zn₆ and Zn₇-MTs as predominant MT species¹¹. While the overall preference for populating non-metalated forms over fully metalated will stem from a faster kinetic of metal binding to apothionein, we speculate that the higher stability of the Cu(I)₄-thiolate cluster in MT-3 and of the Zn(II)₃-cluster in MT-2 will still determine the Cu(I)-thionein nature and observed metalation state in Cu(I)/Zn(II)MT-3.

CONCLUSION

Our work shows that the specific metal-thionein nature (copper or zinc) is controlled and mediated by fine tuning of key non-coordinative residues which increases the copper selectivity and stability in MT-3 β -domain and at the same time decreases its stability toward Zn(II) coordination in a Zn(II)₃-thiolate cluster. The results reveal how the strict interplay between key non-coordinating residues located on both proteins domains control the copper/zinc-thionein character of each MT isoform. Mutation of selected residues in MT-3 to the corresponding amino acids in MT-2 revert this bias resulting in a metalloprotein with increased zinc binding stability and decreased copper binding affinity. Distinct and specific roles can be assigned to these residues in controlling differences in metal exchange kinetics, Cu(I)₄ cluster stability, and/or Zn(II) binding selectivity. Thus, selective mutations allowed us to engineer MT-3 variants in which the copper-thionein character has been converted to zinc-thionein, deepening our understanding on how metal selectivity control can be obtained in MTs metal clusters. It is worth highlighting that all the residues characterized in this study and determined critical for defining the Cu(I) selectivity nature in Cu(I)₄Zn₄MT-3 are also essential residues for maintaining the biological activity in this isoform, establishing an intriguing correlation between metal selectivity bias towards Cu(I) and unique biological activity of MT-3. Determining the 3D structure of a Cu(I)₄Zn(II)₄MT isoform(s) will prove instrumental for understanding at the atomic level how folding and outer sphere effects specifically control the metal selectivity nature in mammalian metallothioneins.

MATERIALS AND METHODS

Recombinant human-MTs expression, purification and metal reconstitution

Recombinant human MT-2, MT-3 and MT-3 mutants were generated by synthetic DNA synthesis upon codon optimization (Genscript Inc.) and cloned in a pET-3d plasmid (Novagen). MTs were expressed in *Escherichia coli* BL21(DE3)pLys following the method of Faller⁵¹. Proteins were expressed and purified as cadmium-bound forms by adding 0.4 mM CdSO₄ 30 minutes after IPTG induction. For the DEAE step, MT-2 and the MT-3 mutants containing the 55–60 deletion were eluted with a linear gradient of 0 to 200 mM NaCl in 25 mM Tris/HCl, pH 8.6, at a flowrate of 8 ml/min using a HiPrep DEAE FF 16/10 column connected to an Äkta Pure chromatographic system (GE Healthcare Life Sciences). The Cd-containing fractions, as determined by ICP-MS quantification which eluted at about 50 mM NaCl, were pooled. MT-3 and the remaining MT-3 mutants were instead eluted with a linear gradient of 100 to 350 mM NaCl in 25 mM Tris/HCl, pH 8.6, and the Cd-containing fractions eluting at about 200 mM were pooled. The apo-proteins were then generated and

reconstituted with zinc using the method of Vasak⁵². The size exclusion chromatography steps after generating the apo-proteins and again after reconstitution to the final Zn₇MTs ensured the complete removal of low molecular weight protein contaminants. Protein concentrations were determined photometrically in 0.1 M HCl using Cary 300 UV-Vis Spectrophotometer (Agilent). For MT-3, $\epsilon_{220} = 53,000 \text{ M}^{-1} \text{ cm}^{-1}$ was used while for MT-2 and the MT-3 mutants, ϵ_{220} were estimated based on the number of peptide bonds using the ϵ_{220} of MT-3 with 67 peptide bonds as the basis. Metal-to-protein ratios were determined by ICP-MS (Agilent 7900) using samples digested in 1% HNO₃. Cysteine-to-protein ratios were measured by quantifying sulfhydryl groups (CysSH) photometrically upon their reaction with 2,2-dithiodipyridine in 0.2 M sodium acetate/1m M EDTA (pH 4.0) using $\epsilon_{343} = 7,600 \text{ M}^{-1} \text{ cm}^{-1}$. Zinc-to-protein ratios of 7.0 ± 0.6 and CysSH-to-protein ratios of 20 ± 3 were obtained. The protein purity was confirmed by SDS-PAGE using MT samples subjected to cysteine modification by monobromobimane following the method of Meloni⁵³.

Reaction of Zn₇MTs with Cu²⁺ followed by UV-Vis Absorption and Stopped-flow spectroscopy

Zn₇MT-3 and Zn₇MT-2 samples (5 μM in 25 mM Tris-HCl/50 mM NaCl, pH 8.0) were rapidly mixed with 4 Cu²⁺ eq. (20 μM CuCl₂ in 20 μM HCl) and incubated for 1 h. Free and loosely bound metals were removed by addition of Chelex 100 (10 mg/ml), followed by centrifugation ($14,000 \times g$, 2.5 min) to remove the resin. UV-Visible absorption spectra were recorded before and after reaction using Cary 300 UV-Vis Spectrophotometer (Agilent) using 1-cm quartz cuvettes. To follow the kinetics of Cu(I) binding, stopped-flow spectrometry using an SX-20 spectrometer (Applied Photophysics) was performed. Equal volumes (70 μl) of 5 μM Zn₇MT in 25 mM Tris-HCl/50 mM NaCl (pH 8.0) and 20 μM Cu²⁺ in 20 μM HCl were simultaneously injected and mixed and the absorbance at 220 nm and 260 nm (10 mm pathlength, 2.325 nm bandpass) were monitored for 601 s at 25 °C. The relative fraction of Zn²⁺ released or Cu⁺ bound to MT at a given time was calculated by determining the fractional differential absorption at 220 nm (at the energy where Zn(II)-CysS LMCT predominate) and 260 nm (at the energy where Cu(I)-CysS LMCT predominate). The fractional Zn(II) bound is reported assuming equal averaged contributions from terminal and bridging thiolates. The total metal exchanged was based on the absorption after 601 s and using the following formulas.

$$\text{Fraction Zn}^{2+} \text{ bound} = \frac{A_{220\text{nm}}(t=i) - A_{220\text{nm}}(t = 601\text{s})}{A_{220\text{nm}}(t = 0\text{s}) - A_{220\text{nm}}(t = 601\text{s})}$$

$$\text{Fraction Cu}^{+} \text{ bound} = \frac{A_{260\text{nm}}(t=i) - A_{260\text{nm}}(t = 0\text{s})}{A_{260\text{nm}}(t = 601\text{s}) - A_{260\text{nm}}(t = 0\text{s})}$$

Determination of Cu,Zn MT stability in air using Size Exclusion Chromatography

Zn₇MTs samples (5 μM in 25 mM Tris-HCl/50 mM NaCl, pH 8.0) were titrated with 0, 4, or 8 equivalents of Cu²⁺ and incubated in aerobic condition for 1 h and 24 h at 37 °C. Free and

loosely bound metals were removed by using Chelex 100 (10 mg/ml) as described. 200 μ l samples were injected into a size-exclusion chromatography (SEC) column (Superdex 75) connected to an Äkta Pure chromatographic system (GE Healthcare Life Sciences) and eluted using 25 mM Tris-HCl/50 mM NaCl (pH 8.0) buffer at a flow rate of 0.8 ml/min. The absorbance was monitored at 220 nm, 235 nm and 260 nm and the protein peaks integrated using Unicorn version 6.4 software (GE Healthcare Life Sciences).

Luminescence characterization of Cu,ZnMT species at 77 K

Low-temperature luminescence spectra and lifetime decays were collected using a FluoroMax-4 spectrofluorometer (Horiba Scientific). Zn₇MT samples (10 μ M) in 25 mM Tris-HCl/50 mM NaCl (pH 8.0) were mixed with four Cu²⁺ equivalents and incubated for 1 h at 25 °C. Free and loosely bound metals were removed using Chelex 100 as described. 400- μ l samples were placed in quartz tubes with 2 mm inner diameter and immersed in cylindrical quartz Dewar filled with liquid nitrogen. Emission spectra (380–750 nm, 5 nm slit) were obtained at 77 K with excitation at 320 nm (5 nm slit), using 10- μ s initial delay and 300- μ s sample window. Lifetime measurements were performed for the emissive bands at 425 nm and 575 nm using 75- μ s initial delay and 300- μ s sample window. 10- μ s and 20- μ s delay increments and 500- μ s and 1000- μ s maximum delays were used for the 425 nm and 575 nm bands, respectively.

Cu(I)-binding competition between MT-2 and MT-3 followed by SEC and ICP-MS

The Cu binding relative affinities of MT-2 and MT-3 were investigated by reacting an equimolar mixture of 5 μ M Zn₇MT-2 and 5 μ M Zn₇MT-3 in 25 mM Tris-HCl/50 mM NaCl (pH 8.0) with 20 μ M Cu²⁺ in 20 μ M HCl for 1 h at 25 °C. Free and loosely bound metals were removed using Chelex 100 as described. 500 μ l of the sample mixture was injected into a size-exclusion chromatography column (Superdex 75) connected to an Äkta Pure chromatographic system (GE Healthcare Life Sciences) and eluted using 25 mM Tris-HCl/50 mM NaCl (pH 8.0) buffer at a flow rate of 0.8 ml/min. The absorbance was monitored at 220, 235 and 260 nm and the protein peaks integrated using Unicorn version 6.4 software (GE Healthcare Life Sciences). Samples from the center of the first peak (corresponding to MT-3, 13 ml) and second elution peak (MT-2, 13.8 ml) were diluted in 1% HNO₃ and analyzed for metal content using ICP-MS (Agilent 7900).

Determination of Cu binding affinity using Bcs

Cu binding affinity determinations were performed in a nitrogen-purged anaerobic glovebox. Samples were made oxygen-free with at least three vacuum/nitrogen cycles on a Schlenk-line. Zn₇MT samples (10 μ M in 25 mM Tris-HCl/50 mM NaCl, pH 7.4) were reacted with four Cu²⁺ equivalents and incubated for 1 h at 25 °C. Free and loosely bound metals were removed using Chelex 100 as described. Bcs (1 mM) was then added and the absorbance of the [Cu(I)L₂]³⁻ complex formed was measured using Cary 300 UV-Vis Spectrophotometer (Agilent) at 483 nm (ϵ_{483} =13,000 M⁻¹ cm⁻¹) after 15 h incubation. The average Cu(I) dissociation constants were calculated according to the following reactions, using the formation constant β_2 =10^{19.8} M⁻² for Bcs³⁶.

$$K_d \beta_2 = \frac{\frac{[P]_{total}}{[P-Cu]} - 1}{\left(\frac{[L]_{total}}{[CuL_2]} - 2 \right)^2 [CuL_2]}$$

Determination of Zn binding affinity using PAR

Zn₇MT samples (1.43 μM in 25 mM Tris/HCl, 50 mM NaCl, pH 7.4) were mixed with 170 μM 4-(2-pyridylazo) resorcinol (PAR) at 25 °C for 2.5 h inside a nitrogen-purged anaerobic glovebox. The concentration of the ZnH_x(PAR)₂ complex formed was then measured at 492 nm (ε=71 500 M⁻¹ cm⁻¹). The dissociation constants K_d^L were calculated according to the following equations, using the effective dissociation constant of ZnH_x(PAR)₂ at pH 7.4 (K_d^{eff} = 7.08 × 10⁻¹³ M²)⁵⁰:

$$K_{ex} = \frac{[H_x PAR]^2 [P-Zn]}{[ZnH_x(PAR)_2] [P]}$$

$$K_d^L = K_d^{eff} \times \frac{1}{K_{ex}}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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This research article is dedicated to the memory of Prof. Silvia Atrian.

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SIGNIFICANCE TO METALLOMIC STATEMENT

The structural, sequence, and reactivity determinants in MTs responsible for metal selectivity bias in metal-thiolate clusters have so far remained elusive. In this work, we dissected and characterized the roles played by isoform-specific conserved non-coordinating residues in controlling metal selectivity in MT-3 and MT-2, which give rise to their unique respective copper-thionein and zinc-thionein characters. The results corroborate at a molecular level how MTs metal selectivity nature underlies fundamental distinct roles played by MT-3 in copper homeostasis and in controlling aberrant copper-protein interactions, and by MT-2 in zinc buffering and homeostasis.

Figure 1.

(A) Schematic representation of the reactivity of Zn_7 MTs towards $Cu(II)$ and products of the reactions investigated in this study. (B) Amino acid alignment of human MT-2A and MT-3 sequences. Conserved metal coordinating cysteines residues are highlighted in yellow and amino acid positions in MT-3 sequence that have been mutated in this work to the corresponding ones in MT-2 are highlighted in light green. An amino acid conservation plot between the two sequences is presented (the scores indicate the degree of similarity between two corresponding position; scheme generated with Jalview).

Figure 2. Electronic absorption spectra of 5 μM $\text{Zn}_7\text{MT-3}$ and 5 μM $\text{Cu(I)}_4\text{Zn}_4\text{MT-3}$ (A), and 5 μM $\text{Zn}_7\text{MT-2}$ and 5 μM $\text{Cu(I)}_4\text{Zn}_4\text{MT-2}$ (B) in 25 mM Tris-HCl/50 mM NaCl, pH 8.0.

Figure 3.

(A) Luminescence emission spectra for Cu(I)₄Zn₄MT-2 (red) and Cu(I)₄Zn₄MT-3 (black) obtained upon reaction of 10 μ M Zn₇MT-2 with 4 Cu²⁺ equivalents in 25 mM Tris/HCl, 50 mM NaCl, pH 8, recorded on frozen samples at 77 K upon excitation at 320 nm. (B) Lifetime determination of the emissive bands at 425 nm and 575 nm in Cu(I)₄Zn₄MT-2 and corresponding fits obtained with a single exponential decay function.

Figure 4.

Size-exclusion chromatograms obtained upon the reaction of 5 μM $\text{Zn}_7\text{MT-2}$ or $\text{Zn}_7\text{MT-3}$ with 0, 4, or 8 Cu^{2+} equivalents incubated aerobically for 1 h or 24 h in 25 mM Tris/HCl, 50 mM NaCl, pH 8.0, recorded at 220 nm (to monitor Zn(II) clusters stability) and 260 nm (to monitor Cu(I) cluster stability).

Figure 5.

Time-dependent kinetic UV absorption spectra of the product of the reaction between 2.5 μM $\text{Zn}_7\text{MT-3}$ in 25 mM Tris-HCl/50 mM NaCl, pH 8.0 and 4 Cu^{2+} equivalents followed for 600 s at 25 $^\circ\text{C}$ (A) and the kinetic traces at 220 nm and 260 nm monitoring Zn(II) release and Cu(I) binding (B) in MT-3.

Figure 6.

Kinetic profile of the reaction of 2.5 μM $\text{Zn}_7\text{MT-2}$ or $\text{Zn}_7\text{MT-3}$ in 25 mM Tris/HCl, 50 mM NaCl, pH 8.0 with 4 Cu^{2+} equivalents monitored at 260 nm (MT-3, red; MT-2 blue) and at 220 nm (MT-3, black; MT-2 gray) for 600 s at 25 °C. The data reveal that Cu(I) binding reaction is > 95% completed after approx. 75 s for MT-3 and 250 s for MT-2.

Figure 7:
Sequence alignment of MT-3 mutants investigated in this work. Amino acids in MT-3 sequence mutated to the corresponding amino acids present in MT-2 sequence are highlighted in light green, while deletions are highlighted in red boxes.

Figure 8.

Luminescence emission spectra for $\text{Cu(I)}_4\text{Zn}_4\text{MTs}$ obtained from the reaction of $10\ \mu\text{M}$ Zn_7MT samples in 25 mM Tris/HCl, 50 mM NaCl, pH 8.0 with 4 Cu^{2+} equivalents, recorded on frozen samples at 77 K upon excitation at 320 nm (a: MT-3; b: T5 MT-3; c: P7SP9A MT-3; d: T5-P7SP9A MT-3; e: E23K MT-3; f: E23KG24E MT-3; g: 55–60 MT-3; h: T5-P7SP9A-E23K MT-3; i: T5-P7SP9A- 55–60 MT-3; j: T5-P7SP9A-E23K- 55–60 MT-3; k: T5-P7SP9A-E23KE24K- 55–60 MT-3; and l: MT-2).

Figure 9. Kinetic traces of the reaction of 2.5 μM $\text{Zn}_7\text{MT-2}$, $\text{Zn}_7\text{MT-3}$, or $\text{Zn}_7\text{MT-3}$ mutants in 25 mM Tris/HCl, 50 mM NaCl, pH 8.0 with 4 Cu^{2+} equivalents monitored at 260 nm at 25 $^\circ\text{C}$.

Table 1.

Average Cu(I) dissociation constants (K_d) of $\text{Cu(I)}_4\text{Zn(II)}_4\text{MT}$ samples determined from competition reactions of 10 μM $\text{Cu}_4\text{Zn}_4\text{MT}$ with 1 mM Bcs at pH 7.4. The values were calculated using the effective molar absorption coefficient $\epsilon_{483\text{nm}} = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Bcs, and the formation constant $\beta_2 = 10^{19.8} \text{ M}^{-2}$ ³⁶.

	Average K_d (M)
MT-3	$5.64 \pm 0.34 \cdot 10^{-20}$
MT-2	$4.32 \pm 0.80 \cdot 10^{-19}$
T5 MT-3	$1.41 \pm 0.31 \cdot 10^{-19}$
P7SP9A MT-3	$1.57 \pm 0.48 \cdot 10^{-19}$
T5-P7SP9A MT-3	$1.53 \pm 0.03 \cdot 10^{-19}$
55–60 MT-3	$1.19 \pm 0.41 \cdot 10^{-19}$
E23K MT-3	$1.32 \pm 0.09 \cdot 10^{-19}$
E23KG24E MT-3	$1.45 \pm 0.16 \cdot 10^{-19}$
T5-P7SP9A- 55–60 MT-3	$2.46 \pm 0.46 \cdot 10^{-19}$
T5-P7SP9A-E23K MT-3	$2.16 \pm 0.06 \cdot 10^{-19}$
T5-P7SP9A-E23K- 55–60 MT-3	$2.81 \pm 0.10 \cdot 10^{-19}$
T5-P7SP9A-E23KE24K- 55–60 MT-3	$2.99 \pm 0.53 \cdot 10^{-19}$

Table 2.

Zn dissociation constants (K_d) of Zn₇MT samples determined from competition reactions of 1.43 μ M Zn₇MT with 170 μ M PAR at pH 7.4. The values were calculated using the effective molar absorption coefficient $\epsilon_{492\text{nm}} = 71,500 \text{ M}^{-1}\text{cm}^{-1}$ and the effective dissociation constant of $\text{ZnH}_x(\text{PAR})_2$, $-\log K_d^{\text{eff}} = 12.15$ ⁵⁰. These values are averaged K_d s of all Zn binding sites.

	K_d (M)
MT-3	$1.29 \pm 0.05 \cdot 10^{-10}$
MT-2	$3.06 \pm 0.12 \cdot 10^{-11}$
T5 MT-3	$1.06 \pm 0.25 \cdot 10^{-10}$
P7SP9A MT-3	$4.98 \pm 0.20 \cdot 10^{-11}$
T5-P7SP9A MT-3	$4.18 \pm 0.05 \cdot 10^{-11}$
E23K MT-3	$1.02 \pm 0.05 \cdot 10^{-10}$
E23KG24E MT-3	$9.77 \pm 0.15 \cdot 10^{-11}$
55–60 MT-3	$1.11 \pm 0.14 \cdot 10^{-10}$
T5-P7SP9A- 55–60 MT-3	$4.21 \pm 0.68 \cdot 10^{-11}$
T5-P7SP9A-E23K MT-3	$4.16 \pm 0.13 \cdot 10^{-11}$
T5-P7SP9A-E23K- 55–60 MT-3	$4.14 \pm 0.38 \cdot 10^{-11}$
T5-P7SP9A-E23KG24E- 55–60 MT-3	$4.23 \pm 1.90 \cdot 10^{-11}$