

Chapter 1

METALLOTHIONEIN STRUCTURE AND REACTIVITY

Milan Vašák and Gabriele Meloni

The structure and chemistry of mammalian metallothioneins (MTs) with divalent (Zn^{II} , Cd^{II}) and monovalent (Cu^{I}) metal ions pertinent to their role in biological systems are discussed. In human, four MT isoforms designated MT-1 through MT-4 are found. The characteristic feature of these cysteine- and metal-rich proteins is the presence of two metal-thiolate clusters located in independent protein domains. The structure of these clusters is highly dynamic, allowing a fast metal exchange and metal transfer to modulate the activity and function of zinc-binding proteins. Despite the fact that the protein thiolates are involved in metal binding, they show a high reactivity toward electrophiles and free radicals, leading to cysteine oxidation and/or modification and metal release. The unusual structural properties of MT-3 are responsible for its neuronal growth inhibitory activity, involvement in trafficking of zinc vesicles in the central nervous system (CNS), and protection against copper-mediated toxicity in Alzheimer's disease. MT-1/MT-2 also play a role in cellular resistance against a number of metal-based drugs.

Keywords: Metallothionein; three-dimensional structure; structure dynamics; metal-thiolate cluster; metal-cluster reactivity; zinc; copper.

1. Introduction

Metallothioneins (MTs) are a superfamily of low-molecular-mass cysteine- and metal-rich proteins or polypeptides conserved through evolution and present in all eukaryotes and certain prokaryotes. They were discovered initially in the search for the tissue constituent responsible for the natural accumulation of cadmium in equine and human kidney in 1957 by Margoshes and Vallee (Margoshes and

Vallee, 1957). This protein was subsequently named “metallothionein” to reflect the extremely high thiolate sulfur and metal content, both of the order of 10% (w/w) (Kägi and Vallee, 1960). Although MTs are still the only macromolecules in which cadmium accumulates naturally, this metal is only one of several optional metallic components of this protein, the others being usually zinc and copper. In fact, in subsequent studies, zinc was found to be the most abundant and often sole metallic component in mammalian tissues under normal physiological conditions. Besides Zn^{II} , Cu^{I} , and Cd^{II} , MTs bind *in vitro* and in certain cases also *in vivo* (e.g. Pt^{II} in cancer chemotherapy) a variety of other metal ions such as Co^{II} , Ni^{II} , Fe^{II} , Hg^{II} , Pt^{II} , Au^{I} , Ag^{I} , In^{III} , Sb^{III} , Bi^{III} , As^{III} , and TcO^{III} (Vašák and Romero-Isart, 2005). At present, it is becoming increasingly clear that MTs fulfill different functions in a number of biological processes. In mammalian cells, these include homeostasis and transport of physiologically essential metals (Zn, Cu), metal detoxification (Cd, Hg), protection against oxidative stress, maintenance of intracellular redox balance, regulation of cell proliferation and apoptosis, protection against neuronal injury and degeneration, and regulation of neuronal outgrowth (reviewed in Palmiter, 1998; Maret, 2000; Miles *et al.*, 2000; Hidalgo *et al.*, 2001). The binding of copper to mammalian MTs plays mainly a role in copper sequestration in copper-related diseases such as Menkes and Wilson’s diseases (Tapiero *et al.*, 2003). MTs also play a critical role in the chemotherapy of certain cancers, both in the development of tolerance to chemotherapeutics and as an adjunct to reduce toxic side-effects (Cherian *et al.*, 2003; Theocharis *et al.*, 2004).

Differential expression of mammalian MT isoforms is tightly regulated during development and in pathological situations (Miles *et al.*, 2000; Theocharis *et al.*, 2004). In mammals, four distinct MT isoforms exist designated MT-1 through MT-4. They are acidic 6–7-kDa proteins possessing a novel type of metal-thiolate cluster. MT-1 and MT-2 are expressed in almost all tissues. Their biosynthesis is inducible by a variety of stress conditions and compounds

including glucocorticoids, cytokines, reactive oxygen species, and metal ions (Miles *et al.*, 2000); whereas MT-3 and MT-4 are relatively unresponsive to these inducers. Although MT-1/MT-2 are cytosolic proteins, during development they have also been detected in the nucleus (Nartey *et al.*, 1987). MT-3, also known as the growth inhibitory factor (GIF), has been discovered as a factor deficient in the brain of Alzheimer's disease patients. The protein occurs intracellularly and extracellularly, and exhibits neuronal growth inhibitory activity in neuronal cell cultures (Uchida *et al.*, 1991). MT-3 expression is primarily confined to the central nervous system (CNS), where it represents a major component of the intracellular Zn^{II} pool in zinc-enriched neurons (ZENs) (Masters *et al.*, 1994). Lower expression levels of MT-3 have also been reported in pancreas, kidney, stomach, heart, salivary glands, organs of the reproductive system, and maternal deciduum (Sogawa *et al.*, 2001; Irie *et al.*, 2004). The expression of the lastly identified mammalian metallothionein isoform, MT-4, has been found restricted to cornified and stratified squamous epithelium (Quaife *et al.*, 1994). The developmentally regulated MT-4 expression in maternal deciduum together with the expression of the entire *MT* gene locus have been reported in mouse (Liang *et al.*, 1996).

2. Gene Structure and Regulation

Based on the classification by Binz and Kägi (1999), the mammalian *MT* gene family belongs to the vertebrate family (family 1), which is characterized by the consensus sequence K-Xaa(1,2)-C-C-Xaa-C-C-P-Xaa(2)-C, where Xaa stands for other amino acids. Within this family, mammalian metallothioneins constitute four different sub-families designated m1 (MT-1) through m4 (MT-4). In *Homo sapiens*, a significant genetic polymorphism exists. The human *MT* genes are located on chromosome 16q13 and are encoded by a multigene cluster of tightly linked genes. There are at least seven functional *MT-1* genes (*MT-1A*, *MT-B*, *MT-E*, *MT-F*, *MT-G*, *MT-H*, and *MT-X*) and a

single gene encoding the other MT isoforms MT-2 (*MT-2A*), MT-3, and MT-4 (Cherian *et al.*, 2003). A number of other *MT* or *MT*-like genes and pseudogenes with a significant homology to functional *MT* genes exist within the human genome, but their functionality is so far unknown. The *MT* genes encode for two-domain proteins. The three exons composing the human *MT* genes sequentially encode for the N-terminal region of the β -domain (exon 1), the rest of the β -domain (from residue 11 or 12; exon 2), and all of the α -domain (from residue 31 or 32; exon 3). Exons 2 and 3 are spliced at the junction of codons for the Lys/Lys or Arg/Lys residues in the interdomain region.

The promoter regions of the *MT-1* and *MT-2* genes contain several metal-responsive elements (MREs) and glucocorticoid-responsive elements (GREs) as well as elements involved in basal level transcription (BLEs). MT expression is also regulated by oxidative stress by antioxidant responsive elements (AREs) or by MREs which are also responsive to oxidants. The metal-regulatory transcription factor (MTF-1), which is essential for basal expression and induction by zinc, binds to proximal promoter MREs. MTF-1 binds to MREs through its six C2H2 zinc fingers (Heuchel *et al.*, 1994). The DNA sequences responsible for cell-specific regulation of *MT-3* and *MT-4* genes are currently unknown. The regulation of tissue-specific *MT-3* gene expression does not appear to involve a repressor; thus, other mechanisms such as chromatin organization and epigenetic modifications may account for the presence or absence of *MT-3* transcription.

3. Structure of Mammalian Metallothioneins

3.1. *Metallothionein-1 and Metallothionein-2*

The metal-free protein, also named apo-metallothionein or thionein, possesses a predominantly disordered structure, which renders it vulnerable to proteolysis (Winge and Miklossy, 1982). However, a well-defined structure develops upon metal binding. The structural features of mammalian metallothioneins were forthcoming almost

exclusively from spectroscopic studies. The first direct evidence for the existence of metal-thiolate clusters in MTs came from the ^{113}Cd nuclear magnetic resonance (NMR) studies of the reconstituted $^{113}\text{Cd}_7\text{MT-2}$ (Otvos and Armitage, 1980). The studies revealed that 20 cysteine residues and 7 divalent metal ions are partitioned between two metal-thiolate clusters, a cyclohexane-like three-metal cluster ($\text{M}_3^{\text{II}}(\text{Cys})_9$) in the N-terminal β -domain (residues 1–30) and an adamantane-related four-metal cluster ($\text{M}_4^{\text{II}}(\text{Cys})_{11}$) in the C-terminal α -domain (residues 31–61). In these clusters, the metal ions are coordinated by both terminal and μ_2 -bridging thiolate ligands (Otvos and Armitage, 1980) in a tetrahedral-type symmetry (Vařák, 1980).

A great deal of knowledge on the chemical features of MT-1/MT-2 pertinent to their function has arisen from the determination of their three-dimensional (3D) structures. The 3D structures of MT-1/MT-2 from various species were obtained mainly by NMR spectroscopy (Arseniev *et al.*, 1988; Schultze *et al.*, 1988; Messerle *et al.*, 1990b; Zangger *et al.*, 1999), but also by X-ray crystallography (Robbins *et al.*, 1991; Braun *et al.*, 1992) (Figs. 1 and 2). The reported 3D structures reveal a similar monomeric two-domain protein of a

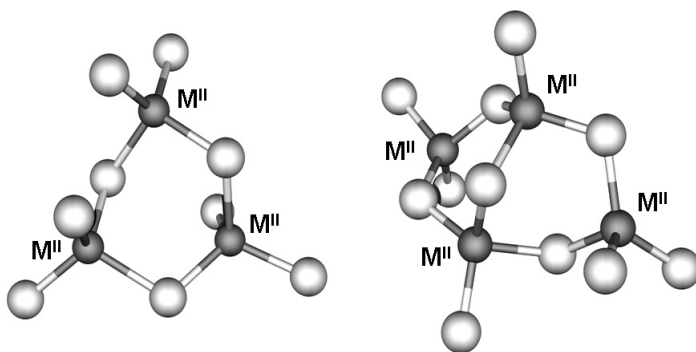


Fig. 1 Schematic drawing of the two metal-thiolate clusters in mammalian $\text{Zn}_2\text{Cd}_5\text{MT-2}$ (Robbins *et al.*, 1991). The metal atoms (M^{II}) are shown as shaded spheres connected to sulfur atoms. The models were generated with the program PyMOL v0.99 (<http://www.delanoscientific.com/>) using the Protein Data Bank (PDB) coordinate 4mt2.

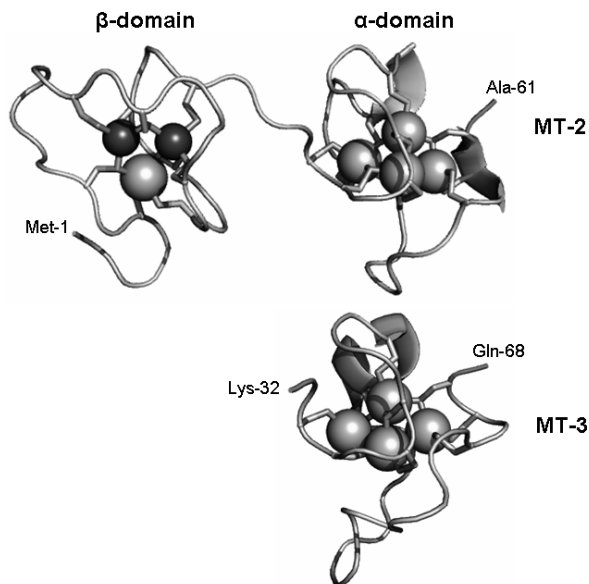


Fig. 2 The three-dimensional crystal structure of rat Zn_2Cd_5MT-2 (top) (Robbins *et al.*, 1991) and the NMR solution structure of the α -domain of human $^{113}Cd_7MT-3$ (bottom) (Wang *et al.*, 2006). The Cd^{2+} and Zn^{2+} ions are shown as shaded spheres connected to the protein backbone by cysteine thiolate ligands. The models were generated with the program PyMOL v0.99 (<http://www.delanoscientific.com/>) using the PDB coordinates 4mt2 and 2f5h.

dumbbell-like shape featuring the cluster topology shown in Fig. 1 and an identical polypeptide folding. The two protein domains in MT-1/MT-2 are connected by a flexible hinge region of a conserved Lys-Lys sequence in the middle of the polypeptide chain.

The flexibility of the protein backbone structure enfolding the metal core in MT-1/MT-2 is well documented. Both the calculated root mean square deviation (RMSD) values from NMR data and the crystallographic B-factors indicate that a considerable degree of dynamic structural disorder exists (Schultze *et al.*, 1988). Direct evidence for the nonrigid MT structure came from the 1H NMR studies of 1H - 2H amide exchange in Cd_7 -MT-1/ Cd_7 -MT-2 (Messerle *et al.*, 1990a; Zangger *et al.*, 1999). In these studies, the enhanced flexibility

of the less structurally constrained β -domain compared to the α -domain in both isoforms has been demonstrated. Molecular dynamics simulations of the β -domain of rat liver MT-2 in aqueous solution also show that the polypeptide loops between cysteine ligands exhibit an extraordinary flexibility without disrupting the geometry of the three-metal cluster (Berweger *et al.*, 2000).

Because of the flexibility of the polypeptide loops between cysteine ligands, it was generally assumed that the protein could accommodate a wide range of divalent metal ions of different sizes without any selectivity. However, in inorganic polynuclear adamantane-like cages, both the metal size and the varying length of the metal-thiolate bonds give rise to widely differing cluster sizes and thus cluster volumes (Hagen *et al.*, 1982). As a consequence of this effect, the binding of different metal ions to MT should lead to an expansion of the cluster core, thus influencing the energetics of the folding process. The metal selectivity of MT-1/MT-2 structures has been studied by offering seven equivalents of two divalent metal ions — i.e. $\text{Co}^{\text{II}}/\text{Cd}^{\text{II}}$, $\text{Zn}^{\text{II}}/\text{Cd}^{\text{II}}$, $\text{Co}^{\text{II}}/\text{Zn}^{\text{II}}$, and $\text{Fe}^{\text{II}}/\text{Cd}^{\text{II}}$ — in a 3:4 ratio to the apoprotein, followed by the determination of the respective metal distributions within and between the clusters by electronic absorption, magnetic circular dichroism (MCD), ^{113}Cd NMR, ^{57}Fe Mössbauer spectroscopy, and electron paramagnetic resonance (EPR) spectroscopy, as required (Good *et al.*, 1991; Pountney and Vašák, 1992). As a result, offering $\text{Co}^{\text{II}}/\text{Cd}^{\text{II}}$ ions resulted in $\text{Co}_3^{\text{II}}\text{Cd}_4\text{MT-2}$ in which two homometallic clusters, i.e. the Co_3^{II} -thiolate cluster in the β -domain and the Cd_4 -thiolate cluster in the α -domain, were present. Conversely, in the case of $\text{Fe}^{\text{II}}/\text{Cd}^{\text{II}}$, the homometallic species $\text{Fe}_7^{\text{II}}\text{MT-1}$ and $\text{Cd}_7\text{MT-1}$ in the ratio of added metal ions were formed. Although $\text{Co}^{\text{II}}/\text{Zn}^{\text{II}}$ and $\text{Zn}^{\text{II}}/\text{Cd}^{\text{II}}$ gave rise to heterometallic clusters in $\text{Co}_3^{\text{II}}\text{Zn}_4\text{MT-1}$ and $\text{Zn}_3\text{Cd}_4\text{MT-2}$, they showed a distinct metal distribution. Thus, an appreciable selectivity in metal partitioning among and within the clusters is indeed observed in this protein. These results are opposite to those obtained in the studies of inorganic adamantane-like cages with monodentate thiolate ligands of the general formula

$[M_4(\text{SPh})_{10}]^{2-}$ ($M = \text{Cd}^{\text{II}}, \text{Zn}^{\text{II}}, \text{Co}^{\text{II}}, \text{Fe}^{\text{II}}$) where, despite the differences in the metal-thiolate affinities and in the homometallic cluster volumes, a simple mixing of two homometallic cages always produced heterometallic cage complexes with an almost statistical distribution of both metal ions (Hagen *et al.*, 1982). Since the same metal ions were employed in the studies of MT-1/MT-2, properties of the MT structure are responsible for this effect. It has been concluded that interplay between both the chemistry of metal ions and the steric requirements of the protein structure determines the diversity of metal-thiolate cluster structures in MT-1/MT-2. In this context, it should be noted that small structural differences between $\text{Zn}_7\text{-MT-2}$ and $\text{Cd}_7\text{-MT-2}$, due to differences in the cluster volumes (approximately 20%), have been observed (Braun *et al.*, 1992).

The coordination of the metal ions is the major determinant in the folding of the polypeptide chain around the two clusters of MT-1/MT-2. The pathway of cluster formation has been determined for $\text{Cd}_7\text{MT-2}$ and $\text{Co}_7\text{MT-2}$ by ^{113}Cd NMR and EPR/paramagnetic ^1H NMR, respectively. Because of the similar size and chemistry of Co^{II} and Zn^{II} , the isostructural substitution of Zn^{II} by Co^{II} is a widely used method to probe the spectroscopically silent zinc sites in proteins. The studies revealed that the formation of both clusters in $\text{Cd}_7\text{MT-2}$ is cooperative and sequential, with the four-metal cluster in the α -domain being formed first (Good *et al.*, 1988). However, the formation of Co^{II} clusters in $\text{Co}_7\text{MT-2}$, and by inference also Zn^{II} clusters, proceeds by a different pathway; in this case, prior to the cluster formation in the α -domain, the first four Co^{II} are bound in independent Co^{II} sites in tetrathiolate coordination (Vašák and Kägi, 1981; Bertini *et al.*, 1989).

At neutral pH, closely similar average apparent stability constants have been determined for MT-1/MT-2 by different methods, with values of the order of 10^{11} M^{-1} and 10^{14} M^{-1} for Zn^{II} and Cd^{II} , respectively (reviewed in Romero-Isart and Vašák, 2002). The recent development of fluorescent metal chelators with different metal affinities allowed the dissection of average apparent stability

constants. Although each of the seven Zn^{II} ions in $\text{Zn}_7\text{MT-2}$ is bound in a tetrathiolate coordination environment, analysis of Zn^{II} binding to thionein revealed at least three classes of binding sites with affinities that differ by four orders of magnitude (Krezel and Maret, 2007). One Zn^{II} ion in $\text{Zn}_7\text{MT-2}$ is relatively weakly bound ($\log K = 7.7$), making MT a zinc donor. Moreover, it has been suggested that physiological ligands, including thionein, would further modulate the metal binding and the redox reactivity of thiols in the cellular environment. These chemical characteristics suggest how the molecular structures and redox chemistries of fully and partially metallated MT and thionein may contribute to the variety of different functions that MT may serve in the cell.

Copper binds readily to MT *in vivo* and *in vitro* (Kägi and Schäffer, 1988). Both Wilson's and Menkes diseases in human are inborn disorders of copper metabolism in which excess copper accumulates intracellularly in MT. In all instances examined to date, copper is bound to MT as Cu^{I} . The only available 3D crystal structure of Cu^{I} -thiolate clusters in MT is that of yeast Cu_8MT . The structure shows the largest known oligonuclear Cu_8^{I} -thiolate cluster in biomolecules, consisting of six trigonally and two diagonally coordinated Cu^{I} ions (Calderone *et al.*, 2005). However, the 3D structure of Cu^{I} containing mammalian MT-1/MT-2 is so far unknown. The structural features of Cu^{I} -thiolate clusters in mammalian MTs have been studied by various spectroscopic techniques (Stillman, 1995). The current knowledge is limited to the fact that fully Cu^{I} -loaded MT-1/MT-2 bind 12 Cu^{I} ions into two metal-thiolate clusters, where, in contrast to divalent metal ions, the monovalent copper ions are coordinated by two or three cysteine ligands forming two independent Cu_6^{I} -thiolate clusters (Nielson *et al.*, 1985; Stillman, 1995). In these studies, the $\text{Zn}_7\text{MT-1}$ form was titrated with Cu^{I} ions. However, the binding studies of Cu^{I} to thionein revealed that, at a lower Cu^{I} /protein stoichiometry, two distinct Cu_4^{I} -thiolate clusters are formed in both protein domains (Pountney *et al.*, 1994). There is evidence to suggest that Cu^{I} binds preferably to the less structurally constrained β -domain (Nielson and

Winge, 1984), and Cd^{II} and Zn^{II} to the α -domain (Nielson and Winge, 1983). A recent NMR investigation of both synthetic domains of MT-1 filled with Cu^{I} or Zn^{II} ions revealed significant differences in their respective polypeptide folds. This observation signifies the different coordination geometries required for the binding of monovalent and divalent metal ions (Dolderer *et al.*, 2007).

3.2. Metallothionein-3

Although MT-1/MT-2 and MT-3 share a conserved array of 20 cysteine residues, evolutionarily conserved changes in the primary structure of MT-3 exist. These are a T₅ insert followed by a unique C₆-P-C-P₉ sequence within the β -domain, and an acidic hexapeptide insert within the α -domain (Fig. 3).

The structural studies on recombinant $\text{Zn}_7\text{MT-3}$ and $\text{Cd}_7\text{MT-3}$ established the presence of two mutually interacting protein domains, resembling those reported for MT-1 and MT-2, with each domain encompassing a metal-thiolate cluster (Faller and Vašák, 1997; Bogumil *et al.*, 1998; Hasler *et al.*, 1998; Faller *et al.*, 1999). The presence of a three-metal cluster in the N-terminal β -domain (residues 1–31) and a four-metal cluster in the C-terminal α -domain (residues 32–68) of Zn^{II} and Cd^{II} containing $\text{M}_7^{\text{II}}\text{MT-3}$ was inferred from the studies of separate α - and β -domains. The metal-binding affinity of MT-3 is weaker than that of MT-1/MT-2, and the metal binding to MT-3 is noncooperative (Hasler *et al.*, 2000; Palumaa *et al.*, 2002). When compared to $\text{Cd}_7\text{MT-1}/\text{Cd}_7\text{MT-2}$, a markedly increased structure flexibility and cluster dynamics exist in $\text{Cd}_7\text{MT-3}$. This information was forthcoming from ^{113}Cd NMR studies of

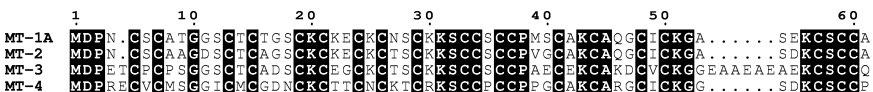


Fig. 3 KALIGN amino acid sequence alignment of four human metallothionein isoforms, with the conserved residues highlighted. The figure was generated with the program ESPript version 2.2.

Cd₇MT-3, in which a significant broadening of all NMR resonances and a very low and temperature-independent intensity of the Cd₃Cys₉ cluster resonances have been interpreted in terms of dynamic processes acting on two different NMR time scales: (1) fast exchange between conformational cluster substates and (2) additional, very slow exchange processes between configurational cluster substates in the β -domain (Faller *et al.*, 1999). The changes in conformational substates may be visualized as minor dynamic fluctuations of the metal coordination environment, and those of the configurational substates as major structural alterations brought about by temporary breaking and reforming of the metal-thiolate bonds. Therefore, only the 3D structure of the α -domain of mouse and human ¹¹³Cd₇MT-3 could be determined by NMR (Fig. 2, bottom). The structure reveals a peptide fold and a cluster organization very similar to those found in Cd₇MT-1/Cd₇MT-2, with the exception of an extended flexible loop encompassing the acidic hexapeptide insert (Öz *et al.*, 2001; Wang *et al.*, 2006).

Since the mutation of conserved proline residues in the T₅CPCP₉ motif of MT-3 to Ala and Ser — the amino acids present in MT-2 — abolished the neuroinhibitory activity and cluster dynamics, it has been suggested that dynamic events centered at the three-metal cluster of MT-3 would include a partial unfolding of the β -domain and that the *cis/trans* interconversion of Cys-Pro amide bonds would govern the kinetics of this process (Hasler *et al.*, 2000). Additional insights into this process were provided by a molecular dynamics (MD) simulation of the β -domain of Cd₇MT-3 (Ni *et al.*, 2007). The studies revealed that, due to the structural constraints introduced by the T₅CPCP₉ motif, an unusual conformation of the N-terminal fragment (aa 1–13) is formed when compared with Cd₇MT-2, and that the formation of the *trans/trans* isomer is energetically more favorable. Further simulation of the partial unfolding supported the proposed role for *cis/trans* interconversion of Cys-Pro amide bonds in the folding/unfolding process of the β -domain. Other studies using the chimeric MT forms, generated by swapping the MT domains of

MT-3 and MT-1, showed that the α -domain — via domain–domain interactions — modulates the bioactivity of the β -domain of MT-3 and that, besides T₅, P₇, and P₉ mutations (Hasler *et al.*, 2000), the E23K mutation also abolishes the growth inhibitory activity of MT-3 (Ding *et al.*, 2006; Ding *et al.*, 2007). Although the mechanisms underlining these effects remain to be elucidated, the results obtained so far suggest that the structure of the β -domain of MT-3 is subjected to a fine tuning. Taken together, the changes in the primary structure of MT-3 in comparison with MT-1/MT-2 result in extraordinary and unprecedented structure dynamics and thus in the alteration of surface topology important for its bioactivity, playing a putative role in protein–protein or protein–receptor interactions.

The specific binding of intracellularly occurring neuronal Zn₇MT-3 to the small GTPase Rab3A in its GDP-bound state has been demonstrated, and the nature of the complex characterized. Rab3A is critically involved in the exo-endocytotic cycle of synaptic vesicles, including neuronal zinc vesicles. This finding indicates that Zn₇MT-3 actively participates in the synaptic vesicle trafficking upstream of vesicle fusion, playing a chaperone, sensor, and/or effector role in this process (Knipp *et al.*, 2005). The identification of MT-3 as a component of a brain multiprotein complex with heat shock protein 84 (HSP84) and creatine kinase (CK) has been linked to the peculiar structural properties of the protein described above (El Ghazi *et al.*, 2006).

As isolated from the brain, MT-3 contains both Cu^I and Zn^{II} ions in Cu₄Zn_{3–4}MT-3. The extended X-ray absorption fine structure (EXAFS) studies of this species revealed the presence of two homometallic clusters: a Zn_{3–4}-thiolate cluster and a Cu₄^I-thiolate cluster with Zn^{II} and Cu^I ions tetrahedrally and trigonally coordinated, respectively (Bogumil *et al.*, 1998). A striking feature of the Cu₄^I-thiolate cluster, which by immunochemical methods was found to be localized in the β -domain (Roschitzki and Vašák, 2002), is its remarkable stability against air oxidation. By contrast, the studies on a well-defined Cu₄Zn₄MT-3 form prepared by a selective metal

reconstitution of both domains *in vitro* revealed the presence of a redox-labile zinc site in the Zn₄-thiolate cluster located in the α -domain. While under anaerobic or reducing conditions the Zn₄-thiolate cluster is stable, a partial oxidation of specific thiolate ligands in air results in Zn^{II} release, giving rise to a Zn₃-thiolate cluster in the α -domain (Roschitzki and Vařák, 2003). It may be noted that the neuroinhibitory activity of MT-3 in cell cultures was found for both Cu₄Zn₃₋₄MT-3 and Zn₇MT-3 metalloforms (Uchida *et al.*, 1991; Erickson *et al.*, 1994). However, comparative biological studies on well-defined metalloforms are currently lacking.

In metal-linked neurodegenerative disorders like Alzheimer's disease, a dysregulated copper homeostasis and related neurotoxicity are linked to the production of reactive oxygen species (ROS). A protective role of extracellularly occurring Zn₇MT-3 against copper-mediated toxicity has been suggested based on its reactivity with free Cu^{II} ions *in vitro*. The studies showed that Zn₇MT-3, through Cu^{II} reduction to Cu^I by thiolate ligands and binding to the protein forming an air-stable Cu(I)₄Zn₄MT-3 species, can efficiently scavenge and redox-silence the toxic free Cu^{II} ions (Meloni *et al.*, 2007).

3.3. Metallothionein-4

The last identified member of the mammalian MT family is MT-4. This isoform consists of 62 amino acids, showing an insert of Glu in position 5 relative to MT-1/MT-2 proteins (Fig. 3). MT-4 appears to be present exclusively in cornified and stratified squamous epithelium. Much of the information on MT-4 deals with gene regulation molecular biology and expression profiles in mammalian maternal deciduum (Liang *et al.*, 1996), and during epithelium development and physiology (Quaife *et al.*, 1994; Schlake and Boehm, 2001). All of these studies revealed that the *MT-4* gene is subject to a strict developmental regulation. However, the question of whether MT-4 is involved in copper or zinc metabolism in epithelia is still debated.

Insights into the function of a protein have been inferred from its structural properties. From the studies of metal-binding abilities of MT-4 using heterologously expressed MT-4 with zinc, cadmium, and copper in combination with the *in silico* protein sequence analyses, the copper-binding nature has been suggested (Tio *et al.*, 2004). However, from biological studies, a special role of MT-4 in the regulation of zinc-dependent processes in keratinocytes has also been proposed (Quaife *et al.*, 1994). This function is supported by the structural studies on Cd₇MT-4 in which similar average apparent metal-binding affinities of two metal-thiolate clusters in Cd₇MT-4 and Cd₇MT-1/Cd₇MT-2, overall similarities in cluster topologies to that determined for Cd₇MT-1/Cd₇MT-2 (Fig. 2), and the pathway of cluster assembly have been found (Meloni *et al.*, 2006). Thus, MT-4 may be involved in both zinc and copper metabolism in keratinocytes.

4. Reactivity of Metal-Thiolate Clusters

Although the thiol groups in MT are masked through their interaction with metal ions, they retain a substantial degree of the nucleophilicity seen with the metal-free protein. This property is reflected by the extremely high reactivity of the coordinated cysteine ligands with alkylating and oxidizing agents such as iodoacetamide or 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), respectively (Shaw *et al.*, 1991). Different sulfur reactivities in both domains have also been reported. Kinetic, mass spectrometric, and NMR studies have shown that the kinetically preferred reaction of mammalian MT with electrophiles may be localized in either the α - or the β -domain, depending on the specific attacking reagent. For instance, whereas iodoacetamide and *p*-(hydroxymercuri)benzoate have been found to react preferentially with the β -domain, DTNB, aurothiomalate, melphalan, and chlorambucil have been shown to react preferentially with the α -domain (Yu *et al.*, 1995; Zaia *et al.*, 1996; Muñoz *et al.*, 1999). Cysteine residues of the zinc-thiolate clusters in Zn₇MT-1/Zn₇MT-2 can

also be oxidized by mild cellular oxidants like oxidized glutathione (GSSG), releasing bound metal ions in this process (Jacob *et al.*, 1998). Experiments in the presence of the GSH/GSSG redox pair provided evidence for an oxidoreductive mechanism modulating the zinc affinity of the cysteine thiolate ligands in Zn₇MT *in vitro* (Jiang *et al.*, 1998; Maret and Vallee, 1998). The importance of redox-active cysteine ligands in zinc proteins, especially Zn₇MTs, in converting redox signals to zinc signals in a cellular environment has also been discussed (Krezel *et al.*, 2007).

Another interesting aspect of MT reactivity is its ability to react with radical species. Thus, it has been shown that mammalian M₇MT (M = Zn^{II} and/or Cd^{II}) are efficient scavengers of the ROS such as hydroxyl ($\bullet\text{OH}$) and superoxide ($\bullet\text{O}_2^-$) radicals or the reactive nitrogen species (RNS) such as nitric oxide ($\bullet\text{NO}$) (Thornalley and Vařák, 1985; Kröncke *et al.*, 1994). In all instances, the free radical attack occurs at the metal-bound thiolates, leading to the protein oxidation and/or modification and subsequent metal release. Interestingly, in many instances, these effects could be reversed under reductive conditions and in the presence of the appropriate metal ion. The protective role of MTs against ROS damage in biological systems is well documented (reviewed in Fabisiak *et al.*, 2002). Although the reactivity of MT-1/MT-2/MT-3 with ROS is comparable, MT-3 can scavenge free NO and NO from *S*-nitrosothiols more efficiently than MT-1 and MT-2. In this process, the zinc-thiolate bonds are targets for both a direct attack by free NO and *S*-nitrosothiols, leading to the Cys*S*-NO formation and metal release. Further reaction of these Cys*S*-NO groups results in the formation of intramolecular disulfide bonds in the MT structure. ¹H and ¹¹³Cd NMR studies of Cd₇MT-1 revealed that, whereas NO selectively releases the metals from the N-terminal β-domain, the C-terminal α-domain is less sensitive to the NO attack (Zangger *et al.*, 2001); in contrast, *S*-nitrosothiols efficiently release zinc from both the α- and β-domains of Zn₇MT-3. The *S*-nitrosylation of MT-3 by *S*-nitrosothiols occurs via a transnitrosation reaction. The increased reactivity of MT-3 with free NO and

S-nitrosothiols led to the proposal that Zn₇MT-3 may specifically convert NO signals into zinc signals (Chen *et al.*, 2002).

Studies on ligand substitution reactivity have revealed that small multidentate ligands (e.g. polyamines, polyaminocarboxylates, bis(thiosemicarbazones)) are effective competitors for zinc bound to MT in reactions which are much faster than the dissociation rate constant for zinc in Zn₇MT-1/Zn₇MT-2, implying a direct competition mechanism (Petering *et al.*, 1992). Biphasic kinetics and differential reactivity of the two metal clusters, α - and β -domains, in mammalian M₇^{II}MT have been measured with ethylenediaminetetraacetic acid (EDTA) showing $\beta > \alpha$ (Gan *et al.*, 1995), and in opposite order $\alpha > \beta$ with nitrilotriacetic acid (NTA) (Li and Otvos, 1998); on the other hand, bidentate ligands such as ethylenediaminediacetic acid and triethylenetetramine were found to be ineffective, even at thermodynamically competent concentrations. From these studies, it has been suggested that a tripod configuration of chelating ligands is required in thiolate substitution and that only specific regions of the protein domains may provide an easy access to the metal clusters. In this context, it may be noted that, in the crystal structure of Zn₂Cd₅MT-2, each domain contains a solvent-exposed cleft containing three accessible cysteine sulfurs. In summary, the reactivity of the MT structure, which is pertinent to its function, is dominated by the chemistry of the nucleophilic thiolate groups.

5. Structure Dynamics

Despite the high thermodynamic stability of the metal-thiolate clusters in MT, and as a consequence of the structure dynamics, they are kinetically very labile, i.e. the thiolate ligands allow a rapid metallation and demetallation. The rate of metal exchange in MT-1/MT-2 follows the order Hg^{II} > Cd^{II} > Zn^{II}. A comparison with similar studies on inorganic complexes affords the most plausible explanation for the kinetics of metal exchange (Martell *et al.*, 1994). In the latter studies, a correlation between the level of ligand

preorganization and complex lability revealed that a decreasing rigidity of the ligand structure results in an increasing lability of the metal complexes. Based on the already-discussed properties of metal-free and metal-containing MTs, the apoprotein with multidentate cysteine thiolate ligands resembles chelating inorganic ligands with long bridges, for which a low level of ligand preorganization and hence a high kinetic lability has been shown. Evidence for the kinetic lability of metals in Cd₇MT was provided by ¹¹³Cd NMR saturation transfer experiments, which established the presence of intermolecular and/or intramolecular metal exchange within the three-metal cluster of the β-domain with a half-life of the order of 0.5 seconds. The occurrence of similar processes taking place within the four-metal cluster, but with a half-life of about 16 minutes, was afforded by metal exchange studies using the radioactive ¹⁰⁹Cd isotope (Otvos *et al.*, 1993). The importance of protein dynamics for metal exchange has been recognized in the NMR studies of Cd₇MT-1 in which the enhanced backbone flexibility, when compared to Cd₇MT-2, resulted in a much faster intersite cadmium exchange in the three-metal cluster (Zangger *et al.*, 1999). In this context, it may be noted that in zinc enzymes such as alkaline phosphatase and carboxypeptidase, where a well-defined catalytic site is present in a rather rigid protein structure, the exchange half-life of zinc is in the order of hours and days. Thus, the actual exchange rates of metal ions in proteins are not an intrinsic attribute of their binding properties, but rather are determined by the energetics and kinetics of protein folding.

Intermolecular zinc transfer between zinc proteins and Zn₇MT *in vitro* has also been studied, leading to the apoenzyme activation and the modulation of zinc-dependent transcription factors (Jacob *et al.*, 1998; Roesijadi *et al.*, 1998). Moreover, Zn₇MT has been shown to be able to transfer one zinc ion to apoenzymes possessing a lower affinity compared to the apparent average binding constant for zinc in Zn₇MT. However, the presence of one weakly bound metal ion in the structure of Zn₇MT has recently been demonstrated (Krezel and Maret, 2007).

6. Metallothioneins in Inorganic Pharmacology

Metallothionein, owing to its affinity to a wide range of metal ions, can through a direct interaction with inorganic drugs influence their efficacy. Among others, the MT-1/MT-2 reactivity with anticancer platinum-based drugs, bismuth drugs, and gold-containing drugs used in rheumatoid arthritis has attracted particular interest. Platinum drugs are effective chemotherapeutic agents for the treatment of testicular cancer and are used in combination therapy for a variety of other tumors. However, the occurrence of intrinsic resistance in some tumors, and that acquired after initial treatment, are the major drawbacks of these chemotherapeutics. Among other responses leading to the resistance, the high reactivity of platinum compounds with the major intracellular thiols glutathione (GSH) and MT confer resistance to these drugs. However, the cysteine ligands in Zn_7MT react with cisplatin much faster than the thiol group in GSH. As a consequence, in an anticancer treatment with platinum-based electrophilic antineoplastic drugs including cisplatin, an overexpression of MTs in cancer cells is observed. A direct interaction of these drugs with MTs is believed to be the primary cause of tumor cell resistance (Kelley *et al.*, 1988). A detailed study on the interaction of Zn_7MT-2 with a number of *cis*- and *trans*- Pt^{II} compounds, including a new-generation Pt^{II} drug with potential use in the clinic, showed that all ligands in *cis*- Pt^{II} compounds, including cisplatin, are replaced by cysteine thiolates of the protein, thereby fully deactivating the drug (Knipp *et al.*, 2007). In contrast, the protein-bound *trans*- Pt^{II} compounds retained their N-donor ligands, thus remaining in a potentially active form; however, no *trans*- Pt^{II} transfer from MT-2 to plasmid DNA was observed. During the binding process of Pt^{II} , the corresponding amount of Zn^{II} is released from MT-2. The binding of released zinc to the metal-responsive transcription factor (MTF-1), which activates MT-2 expression, represents an unrecognized aspect contributing to the cellular resistance against platinum-based anticancer drugs.

The preinduction of MT-1/MT-2 by bismuth compounds, which induce these proteins almost exclusively in the kidney, can reduce the renal toxicity of cisplatin. In addition, bismuth complexes are also used as antiulcer drugs. Bi^{III} binds strongly to MTs, forming $\text{Bi}_7^{\text{III}}\text{MT}$. EXAFS studies of $\text{Bi}_7^{\text{III}}\text{MT}$ revealed that the metal coordination sphere is composed of three to four sulfur atoms with some additional oxygen atoms. This suggests that a widely different MT structure with Bi^{III} ions exists when compared with those reported for Zn^{II} - and Cd^{II} -containing MTs.

It has been suggested that MT, besides playing a possible role in the detoxification of gold drugs in kidney, may also contribute to the retention and localization of gold in the tissues during rheumatoid arthritis treatment with Au^{I} compounds. Moreover, a high content of MT in cells is apparently responsible for their resistance to the growth inhibitory effects of Au^{I} -auranofin (a rheumatoid arthritis drug). The acquired resistance to the gold drug aurothiomalate may be due in part to MT induction by a mechanism described above for Pt^{II} compounds. The structural studies of gold-containing MT, upon the reactions of $\text{M}_7^{\text{II}}\text{MT}$ with gold thiomalate (AuSTm), revealed that an excess of Zn_7MT results in the formation of an $\text{Au}^{\text{I}}(\text{SCys})_2$ complex in which the thiomalate ligand is replaced by the thiolate ligand. However, an excess of AuSTm gave rise to a monodentate $\text{Au}^{\text{I}}\text{SCys}$ coordination and the retention of the thiomalate ligand, i.e. CysS-Au-STm (Laib *et al.*, 1985). Furthermore, mass spectrometry measurements revealed that a gold-binding mechanism to MT is similar to that reported for the formation of gold nanoclusters (Mercogliano and DeRosier, 2006).

Structural information on other metal derivatives of MT, such as Ni^{II} , Fe^{II} , Hg^{II} , Ag^{I} , In^{III} , Sb^{III} , As^{III} , and TcO^{III} , is also available (Vašák and Romero-Isart, 2005). In view of an increasing number of new metal-based drugs for cancer chemotherapy and treatment of other pathologies, with some drugs already in pre-clinical or clinical testing, further studies of MT reactivity with the metal-based drugs are required to shed light on the chemical and

biological rationale behind the mode of action and efficacy of these compounds.

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