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MINIREVIEW

Chemistry and biology of mammalian metallothioneins

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Abstract Metallothioneins (MTs) are a class of ubiquitously occurring low molecular mass, cysteine- and metal-rich proteins containing sulfur-based metal clusters formed with Zn(II), Cd(II), and Cu(I) ions. In mammals, four distinct MT isoforms designated MT-1 through MT-4 exist. The first discovered MT-1/MT-2 are widely expressed isoforms, whose biosynthesis is inducible by a wide range of stimuli, including metals, drugs, and inflammatory mediators. In contrast, MT-3 and MT-4 are noninducible proteins, with their expression primarily confined to the central nervous system and certain squamous epithelia, respectively. MT-1 through MT-3 have been reported to be secreted, suggesting that they may play different biological roles in the intracellular and extracellular space. Recent reports established that these isoforms play an important protective role in brain injury and metal-linked neurodegenerative diseases. In the postgenomic era, it is becoming increasingly clear that MTs fulfill multiple functions, including the involvement in zinc and copper homeostasis, protection against heavy metal toxicity, and oxidative damage. All mammalian MTs are monomeric proteins, containing two metal–thiolate clusters. In this review, after a brief summary of the historical milestones of the MT-1/MT-2 research, the recent advances in the structure, chemistry, and biological function of MT-3 and MT-4 are discussed.

Keywords Zinc · Copper · Mammalian metallothioneins · Metal–thiolate clusters · Neurodegenerative diseases

Introduction

In mammals, four distinct metallothionein (MT) isoforms designated MT-1 through MT-4 exist. The first MT, later found to be composed of the MT-1 and MT-2 isoforms, was discovered more than 50 years ago by Margoshes and Vallee [1] followed by the discovery of MT-3 [2] and MT-4 [3] at the beginning of 1990s. The most predominant isoforms MT-1/MT-2 are expressed in almost all tissues and their biosynthesis is inducible by a variety of stress conditions and compounds, including glucocorticoids, cytokines, reactive oxygen species (ROS), and metal ions [4]. In contrast, MT-3 and MT-4 are relatively unresponsive to these inducers and their expression is primarily confined to the central nervous system (CNS) [5] and cornified and stratified squamous epithelium [3], respectively. Presently, there are a great number of reviews covering different biological and structural aspects of MT research and focusing predominantly on MT-1/MT-2

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[6–16]. In recent years the role of MTs in the brain, in particular of the MT-3 isoform, has been the subject of intense research. The biological studies on brain MTs are reviewed in this special issue. In this contribution, we briefly summarize the historical milestones of MT-1/MT-2 research till about 1990 and focus on the recent advances in the structure, chemistry, and biological functions of MT-3 and MT-4.

Metallothionein-1/metallothionein-2: historical highlights

As discussed by the late Bert L. Vallee [17] in his contribution to the first MT meeting, the discovery of MT has been closely linked with the search for biological material containing cadmium. The first report on the presence of this metal in human organs, in particular kidney, was in the Russian literature in 1941 [18]. In the mid-1950s the translated article became available to Western scientists. Upon screening for cadmium content in kidneys of a few mammalian species, Margoshes and Vallee [1] identified protein containing besides cadmium also a substantial amount of zinc and lesser amounts of copper and iron after fractionation of horse kidney cortex. The purification and characterization of this material by Kägi and Vallee [19] revealed a low molecular mass 6–7-kDa metal- and cysteine-rich protein, named "metallothionein" (MT) to reflect the extremely high thiolate sulfur and metal content, both of the order of 10% (w/w). Further chromatographic separation revealed the presence of two principal MTs, later designated as MT-1 and MT-2, of 61–62 amino acids, of which 20 were cysteines and none of which were aromatic amino acids. Both MTs contained seven Zn(II) or Cd(II)/Zn(II) ions. The subsequent protein sequencing showed a single polypeptide chain with distinct clustering of the 20 cysteine residues into Cys–X–Cys, Cys–Cys and Cys–X–X–Cys sequences, where X stands for amino acids other than cysteine [20] (Table 1). These unique features of the classical mammalian MT-1 and MT-2 isoforms attracted the interest of scientists from fields as different as biochemistry, molecular biology, toxicology, and structural biology.

The toxicology studies were initiated by the finding that MT was present in increased amounts in livers of rabbits exposed to cadmium. It has been postulated that this observation reflects the induced biosynthesis of this protein by this metal and its role in metal detoxification [22]. This initial assumption of MT-1/MT-2 induction by heavy metals was later confirmed by the increased MT content in liver, kidney, and intestines following the parenteral or dietary administration of cadmium, copper, or zinc to experimental animals [23]. In the years since this publication, a large number of articles on the role of MT-1/MT-2 in metal toxicity have been published and similar conclusions have been drawn. However, the real advances were the observations that mice that cannot synthesize any MT are sensitive to cadmium toxicity, whereas mice that express excess amounts of any MT are resistant to this metal [24–26]. It has been demonstrated, moreover, that selection for cadmium resistance with mammalian cells always results in up to 80-fold increase of the entire MT locus [27]. These observations reflected the original discovery of cadmium-containing MT in horse kidney [1]. Even though cases of cadmium toxicity are known, they are rare and exclusively caused by man. Therefore, it has been suggested that even though a characteristic phenotype of cells and mice with altered expression of MTs is the sensitivity to cadmium toxicity, it seems unlikely that the evolutionary conservation of these ubiquitous, inducible genes in most organisms is driven by the ability of MTs to detoxify cadmium [28]. Thus, the current notion is that cadmium detoxification is a property of MTs rather than its evolutionary function [29].

Molecular biology studies on MTs aimed at the understanding of the gene structure, function, and regulation. The genes for human and mouse MT-1/MT-2 have been most extensively studied [30–33]. Whereas four *MT* genes are present in the mouse genome, in human a significant genetic polymorphism exists. The human *MT* genes are located on band 13 of the long arm of chromosome 16 and are encoded by a multigene cluster of tightly linked genes. There are at least seven functional *MT-1* genes and a single gene encoding the other MT isoforms *MT-2* (MT-2A), *MT-3*, and *MT-4* [13]. A number of other *MT* or *MT*-like genes and pseudogenes with significant homology to functional *MT* genes exist within the human genome, but their functionality is unknown. The *MT* genes encode for two-domain proteins. The three exons composing the

Table 1 Alignment of amino acid sequences of the four human metallothionein (MT) isoforms using ClustalW

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          1       10        20        30        40        50        60        68
MT-1H   MDPN-CSCEA GGSCACAGSC KCKKCKCTSC KKSCCSCCPL GCAKCAQGCI CKGA------ SEKCSCCA
MT-2A   MDPN-CSCAA GDSCTCAGSC KCKECKCTSC KKSCCSCCPV GCAKCAQGCI CKGA------ SDKCSCCA
MT-3    MDPETCPCPS GGSCTCADSC KCEGCKCTSC KKSCCSCCPA ECEKCAKDCV CKGGEAAEAE AEKCSCCQ
MT-4    MDPRECVCMS GGICMCGDNC KCTTCNCKTC WKSCCPCCPP GCAKCARGCI CKGG------ SDKCSCCP
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The conserved cysteine residues are highlighted. The numbering is that of the human MT-3 sequence. Generated with SeaView [21]

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 *MT-1* and *MT-2* genes contain several metal-responsive elements (MREs) and glucocorticoid-responsive elements as well as elements involved in basal level transcription. The *MT-1*/*MT-2* expression is also regulated by oxidative stress by antioxidant responsive elements or by MREs that are also responsive to oxidants. Metal-regulatory transcription factor 1 (MTF-1), which is essential for basal expression of MT-1/MT-2 and induction by zinc, binds to promoter proximal MREs. MTF-1 binds to MREs through its six C2H2 zinc fingers [34]. Although similar MREs were also identified in the *MT-3* gene, there were unresponsive to zinc [35]. 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 in humans 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.

In early biochemical studies the biological role of MT-1/MT-2 was sought. As the major zinc-binding proteins in the cell, it has long been hypothesized that MTs can potentially modulate many important biological processes that involve zinc-requiring enzymes. Their involvement can either be direct, via interaction with inactive apoenzymes, or indirect, by regulation of available Zn(II) in the cell. The first demonstration that several zinc-requiring apoenzymes can be reactivated by the transfer of Zn(II) from Zn_7MTs in vitro was 30 years ago [36]. Evidence that apoMT, or thionein, readily removes Zn(II) from zinc-finger transcription factors such as Sp1 and *Xenopus laevis* TFIIIA in vitro, eliminating their DNA binding competence, has also been provided [37, 38]. The in vitro demonstration that MTs are extraordinarily efficient scavengers of free hydroxyl radicals formed during various forms of oxidative stress suggested that these proteins may be a part of such a protective system [39]. The primary targets of the free-radical attack in MTs are thiolate groups of the metal–thiolate clusters, which may be repaired in the cell by reduced glutathione. Following this discovery, evidence supporting such a physiological role of MTs in the cellular defense system against oxidative stress and their induced biosynthesis under these conditions was provided [40]. In the postgenomic era it became clear that MTs can have multiple biological functions.

In view of the high cysteine and metal content, the structural investigations of MT-1/MT-2 were highly challenging. Since most of these studies were conducted before 1990, no recombinant protein expression systems were available. The native MTs, as isolated from livers of experimental animals, contained either Zn(II) or both Zn(II) and Cd(II) ions. To examine the geometry and the organization of metal binding sites, the native metal ions were replaced by paramagnetic Co(II) or by the NMR-active ^{113}Cd isotope ($I = 1/2$). The optical and magneto-optical (magnetic circular dichroism) studies of Co_7MT provided evidence for a tetrahedral-type of geometry of all metal binding sites and their coordination by cysteine thiolates [41]. Convincing evidence for the two-cluster arrangement of the seven divalent metal ions in MT, i.e., for a three-metal and a four-metal cluster, has been provided by homonuclear ^{113}Cd NMR decoupling studies of $^{113}Cd_7$MT. In these studies, the Cd(II) coordination by both terminal and bridging thiolate ligands was inferred from the chemical shift position of the ^{113}Cd resonances [42]. The observation of an antiferromagnetic coupling between metal centers in electron spin resonance and magnetic susceptibility measurements of Co_7MT lend further support for a cluster structure in MTs [43]. Through enzymatic cleavage of Cd_7MT and the ^{113}Cd NMR characterization of the Cd_4-containing peptide, the indication for a two-domain structure, each domain encompassing a metal-thiolate cluster, was obtained [44]. The mechanism of metal binding to apoMT-2 was studied by ^{1}H NMR and magnetic susceptibility measurements of the paramagnetic Co(II) metalloform of the protein at room temperature. The results revealed a detailed binding scheme in which both metal–thiolate clusters in Co_7MT-2 were formed in a noncooperative manner [45]. However, in similar studies using Cd(II), a specific cooperative binding to the α-domain followed by the β-domain was revealed by circular dichroism, magnetic circular dichroism, and ^{113}Cd NMR measurements [46, 47]. The substantially different metal sizes of Cd(II) and Co(II), the latter being closely similar to that of Zn(II), thiolate affinities, and the corresponding bond lengths may account for the differences in the pathway of cluster formation.

Since early attempts to crystallize MT-1/MT-2 failed in a number of crystallography groups, a new solution NMR method for the 3D structure determination, developed by Kurt Wüthrich and coworkers in Zurich, was applied. The first structural studies of Wüthrich's group were conducted on Cd_7MT-2 from rabbit liver. However, the structure elucidation by NMR measurements turned out to be more difficult than expected because of the heterogeneity of native protein, the absence of aromatics, leading to very poor chemical shift dispersion, and the presence of Cd–Cys coordinative bonds in the clusters which could not be analyzed with nuclear Overhasuer effects. Crucial to the structure elucidation was the preparation of structurally homogeneous $^{113}Cd_7$MT-2 samples, generated through the

method of metal reconstitution, and the development of heteronuclear ^{1}H–^{113}Cd correlation experiments enabling the cadmium–cysteine topology of the clusters in the rabbit protein to be unraveled [48].

At about the time of the publication of the cluster topology in rabbit $^{113}Cd_7$MT-2, the crystal structure of native rat Cd_5,Zn_2MT-2 was reported [49]. However, the sequence-specific metal-cysteine coordinative bonds in the crystal structure of the rat protein were substantially different from those found by NMR measurements for the rabbit protein. Given the amount of this covalent connectivity information, it was quite clear that this discrepancy between the two sets of results was not something that could be explained easily. Among others, the most likely reasons considered by the scientific community were (1) the NMR and crystallography results might both be correct as differences in the respective primary structures exist, i.e., the rat protein shows ten substitutions of non-cysteine amino acids, (2) the structural differences were caused by the metal reconstitution of the rabbit protein, and (3) the results obtained using the newly developed NMR technique are presumably incorrect. It is hard to imagine a less desirable event at the early stage in the development of the methodology for NMR structure determination of proteins than that the NMR method should produce a high-profile mistake. Also the structure of $^{113}Cd_7$MT was one of the first protein structures determined by NMR measurements, and it was the first structure of a metalloprotein to be determined. In an effort to resolve the discrepancy, NMR studies aiming at the cluster topology in metal-reconstituted rat $^{113}Cd_7$MT-2 and native rat $^{113}Cd_5$,Zn_2MT-2, generated by the administration of ^{113}Cd(II) salt to experimental animals similarly to samples used in crystallization, were undertaken [50]. The NMR data obtained for the rat protein confirmed the cluster topology seen already with the rabbit protein (Fig. 1). Subsequently the NMR structures of $^{113}Cd_7$MT-2 from rat and rabbit livers were completed [51, 52] and those of human $^{113}Cd_7$MT-2, and, for comparison, native Zn_7MT-2 were also determined [53, 54]. The NMR structures of these three homologous MTs are virtually identical, and later the crystal structure of rat Cd_5,Zn_2MT-2 was redetermined and found to coincide very closely with the NMR structure (Fig. 2) [55, 56].

The determination of the structure of mammalian and later also of the crustacean and echinoderm MTs established the presence of trinuclear and tetranuclear thiolate clusters with divalent metal ions as the characteristic structural motif. These structural studies led to the notion about this class of proteins which include the exclusive metal coordination by cysteines, the absence of aromatic amino acids (with rare exceptions) and secondary structure. However, in recent years the biochemical characterization of bacterial and plant MTs, which differ considerably from mammalian proteins, revealed that besides high cysteine content, also aromatic amino acids, including histidine, are present. Furthermore, structural studies on these MTs revealed that apart from the trinuclear and tetranuclear clusters, also mononuclear and binuclear metal binding sites with divalent metal ions occur. In addition, in these structures a well-defined protein fold and, in some instances, metal coordination by both cysteine and histidine residues are encountered. The structural and functional properties of bacterial and plant MTs are reviewed in this special issue.

Metallothionein-3

Biological properties of metallothionein-3

In the human brain the MT-1/MT-2 and MT-3 isoforms occur [9]. The most distinctive biological property of MT-3 is its extracellular growth inhibitory activity in neuronal primary cultures, which led to its discovery [5]. Thus, MT-3, but not MT-1/MT-2 antagonizes the ability of Alzheimer disease brain extract to stimulate survival and neuritic

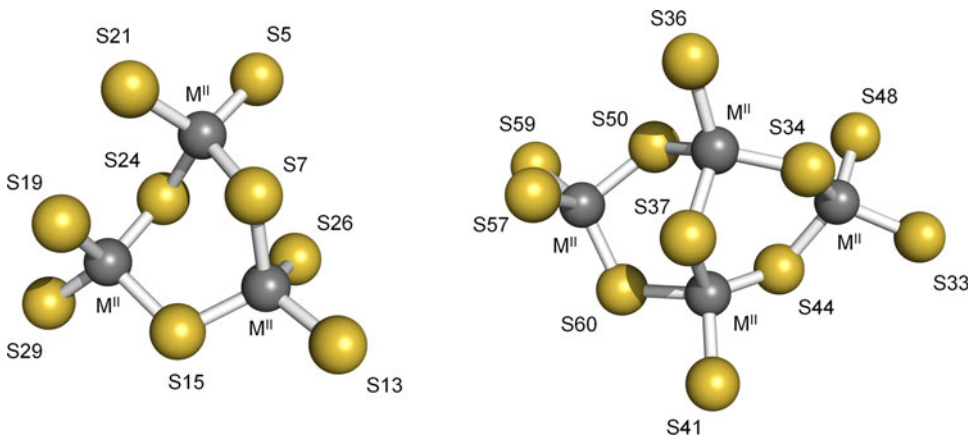

Fig. 1 Structure of the two metal–thiolate clusters with divalent metal ions (*gray spheres*) in mammalian M^{II}_7MT-2. The sulfur atoms (*yellow spheres*) of cysteine thiolates are labeled with the cysteine residue number

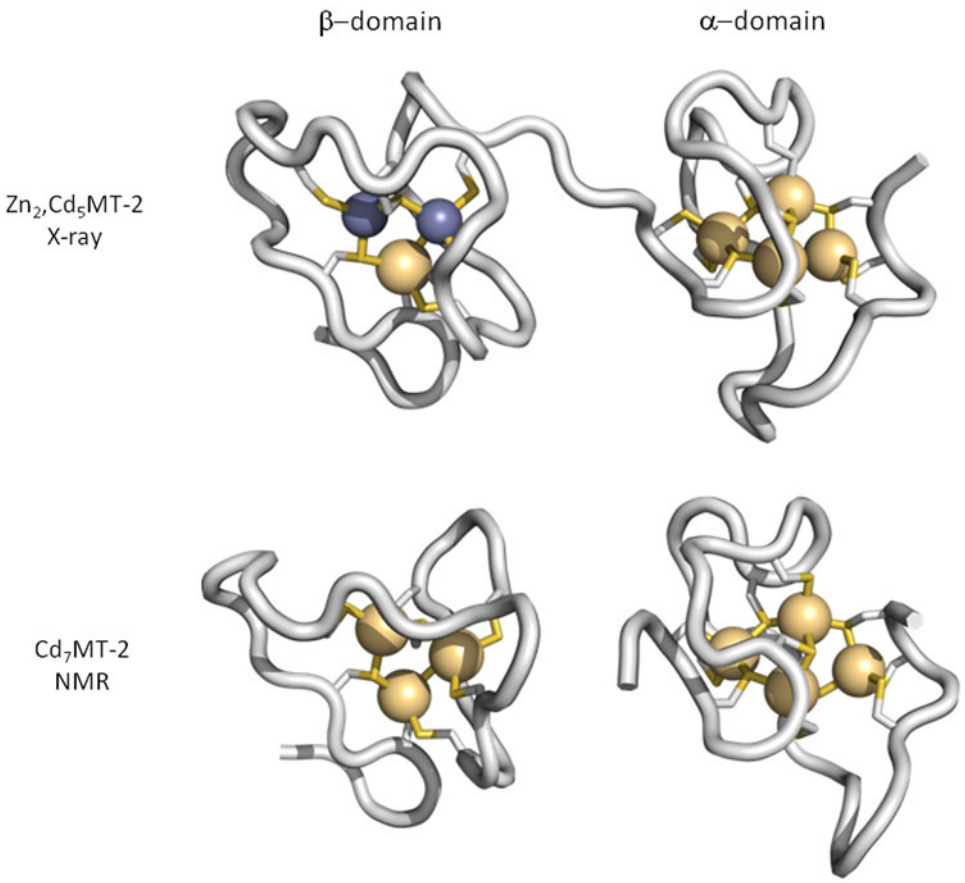

Fig. 2 The three-dimensional X-ray crystal structure of rat Zn_2Cd_5MT-2 (*top*) [55] and the NMR solution structure of the α-domain and β-domain of rat $^{113}Cd_7$MT-2 (*bottom*) [51]. The Cd(II) and Zn(II) ions are shown as *light-orange spheres* and *blue spheres*, respectively, connected to the protein backbone by cysteine thiolate ligands. The models were generated with PyMOL using the Protein Data Bank coordinates of 4MT2, 1MRT, and 2MRT

sprouting of cultured neurons [5, 57]. The observation that constitutive expression of MT-3 but not MT-1 inhibited the growth of cultured kidney cells under zinc-deficient conditions supports distinct functions of both isoforms [58]. The discovered bioactivity led to the hypothesis that MT-3 may be involved in pathogenic processes leading to Alzheimer disease. Independent studies supporting its role in Alzheimer disease showed that MT-3 but not MT-1/MT-2 protects neuronal cells from the toxic effect of amyloid-β (Aβ) peptide Aβ_{1-40} [59]. However, these two effects are functionally unrelated (see below).

That MT-3 displays biological properties not observed for MT-1/MT-2 is clearly documented by in vivo studies in which mice overexpressing MT-3 in most organs died as a result of pancreatic atrophy, whereas expression of similar amounts of MT-1 had no effect [60]. Although the reason for MT-3 toxicity is unknown, these results provide biological evidence that the MT isoforms have different functional properties. Other in vivo studies supporting this conclusion showed that in a mouse model of brain injury, exogenously administered MT-3, in contrast to human MT-2, does not affect inflammation, oxidative stress, and apoptosis [61]. The latter results highlight specific and divergent roles of exogenous MT-3 when compared with the MT-1/MT-2 isoforms.

Numerous studies investigated the localization of MT-1/MT-2 and MT-3 in the CNS. MT-1 and MT-2 are present throughout the brain and spinal cord, with the astrocytes being the main cell type expressing both isoforms. In contrast, MT-3, the isoform predominantly expressed in the CNS [5], is localized in both neurons and astrocytes [10, 62, 63], where it appears to play an important role in the homeostasis of copper and zinc [9]. The MT-1/MT-2 and MT-3 isoforms have been reported to be secreted, suggesting that they may play different biological roles in the intracellular and extracellular space [64–66]. Previously, the involvement of the receptor megalin in the renal uptake of MT-1/MT-2 was described [67]. The same receptor also mediates the transport of MT-1/MT-2 into neurons following brain injury [66]. Presently, no information on whether the megalin receptor also mediates the transport of MT-3 is available.

MT-3 is present in several brain regions at different concentrations, being particularly concentrated in presynaptic terminals of zinc-enriched neurons of cerebral cortex and hippocampus. Zinc-enriched neurons belong to a subset of glutamatergic neurons and as such contain presynaptic zinc vesicles in which 10–15% of the total Zn(II) in the brain is present. The colocalization of MT-3 and zinc vesicles in zinc-enriched neurons led to the suggestion that

the protein may contribute to the utilization of Zn(II) as a neuromodulator [68]. Studies of Zn_7MT-3 and zinc transporter 3 knockout mice revealed that zinc transporter 3, a transporter that concentrates Zn(II) in presynaptic vesicles, and MT-3 function in the same pathway [69]. From these studies an important role for MT-3 in the recycling of Zn(II) was suggested [70]. The demonstration of the direct interaction of Zn_7MT-3 with Rab3A, a small GTPase involved in the regulation of the synaptic vesicle cycle, supports this role [71]. The immunochemical identification of MT-3 as a component of a brain multiprotein complex with heat shock protein 84 and creatine kinase [72] suggests that MT-3 may have additional intracellular functions that remain to be elucidated. Recently, the specific and reversible binding of one extra Zn(II) to Zn_7MT-3 ($K_{app} \sim 100$ μM), forming Zn_8MT-3, was reported [73]. Since during synaptic signaling the free Zn(II) concentration can reach up to 300 μM [74], a reversible switch between the Zn_7MT-3 and Zn_8MT-3 forms may play a role as a zinc buffer or in an interaction with a binding partner(s). The analytical results of the latter study suggest that the reported coexistence of MT-3 species in solution with a metal distribution between five to nine M(II) per protein (M is Zn, Cd), detected by electrospray ionization (ESI) mass spectrometry (MS) upon binding of seven M(II) equivalents to apoMT-3, seems to reflect the reconstitution and ionization conditions used [75].

MT-3, like other MTs [39, 40, 76, 77], efficiently scavenges ROS. Thus, exogenous MT-3 prevents neurite extension and the death of differentiated cortical neurons caused by exposure to high oxygen concentrations, owing to its more efficient scavenging of free hydroxyl radicals than the same concentration of Zn_7MT-1/MT-2 [64]. However, studies of *MT-3*-null mice following damage caused by a focal cryolesion onto the cortex showed that, in contrast to MT-1/MT-2, MT-3 is unlikely to be a significant factor for controlling the inflammatory response, oxidative stress, and apoptosis after significant brain damage, but it may influence neuronal regeneration [78]. The in vitro reactions of *S*-nitrosothiols and H_2O_2 with Zn_7MT-1/MT-2 and Zn_7MT-3 revealed that whereas Zn_7MT-3 was significantly more reactive than Zn_7MT-1/MT-2 toward *S*-nitrosothiols, the reactivity of all three isoforms toward H_2O_2 was comparable. The increased reactivity of Zn_7MT-3 with free NO and *S*-nitrosothiols led to the proposal that Zn_7MT-3 may specifically convert NO signals to zinc signals [79]. The metal-induced aggregation of Aβ and accompanied oxidative stress contribute to the progression of Alzheimer disease (see below). In vitro studies showed that in contrast to amorphous aggregates of Aβ formed upon Zn(II) addition, the Zn(II) release from Zn_7MT-3 upon a slow cysteine oxidation by H_2O_2 induces fibrillar-type Aβ aggregates, thereby modulating its morphology. Thus, the protein may protect cells not only by its capacity for ROS scavenging, but also through Zn(II) release and subsequent binding to Aβ [80]. Recently, in view of the finding that MT-3 regulates lysosomal functions, possible roles of labile zinc and Zn_7MT-3 in oxidative-stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes have been described [81]. Given the accumulating evidence that lysosomes play key roles in many neurological diseases, the role of labile zinc and Zn_7MT-3 in lysosomal function warrants further study.

As discussed above, the regulation of the basal MT-3 expression is poorly understood. Nevertheless, in recent years overexpression of MT-3 in certain cancer types including prostate [82] and breast [83] has been described. Since the *MT-3* gene was identified as hypoxia-inducible in several human tissues [84], the overexpression of MT-3 observed in several cancers presumably reflects the hypoxic conditions, due to insufficient vascularization of the fast-proliferating cancer tissue. MT-3, similarly to MT-1/MT-2, may confer cellular resistance in platinum-based chemotherapy in these cell types [85]. However, kinetics of the reaction of cisplatin with Zn_7MT-3 and Zn_7MT-2 revealed a substantially higher reactivity of cisplatin toward Zn_7MT-3 than Zn_7MT-2 [86].

Structure of metallothionein-3 with divalent metal ions

The distinct biological activity of MT-3 has been linked to the structural differences between MT-1/MT-2 and MT-3. As the structure of MTs is determined by the interplay between the polypeptide chain and metal ions, the differences in amino acid sequences ought to be responsible for the bioactivity. Compared with the amino acid sequences of mammalian MT-1/MT-2 (Table 1), the MT-3 sequence shows two inserts, an acidic hexapeptide in the C-terminal region and a threonine in position 5 in the N-terminal region followed by a conserved C_6PCP_9 motif. Structural information on the metal–thiolate clusters in M^{II}_7MT-3 was forthcoming from spectroscopic investigations of the recombinant protein and chemically synthesized single protein domains. The studies revealed the presence of two mutually interacting protein domains, with each domain encompassing a metal–thiolate cluster [87–90]. A three-metal cluster is located in the N-terminal β-domain (residues 1–31) and a four-metal cluster is located in the C-terminal α-domain (residues 32–68) of M^{II}_7MT-3 [88, 89], a metal organization found also in M^{II}_7MT-1/MT-2 (Fig. 2). From ^{113}Cd NMR studies of human $^{113}Cd_7$MT-3, evidence for unprecedented dynamic processes within the metal–thiolate clusters was obtained [90]. From significant broadening of all ^{113}Cd resonances and the very low and temperature-independent intensity of the Cd_3Cys_9 cluster resonances, the presence of dynamic processes acting on

two different ^{113}Cd NMR timescales was suggested: (1) fast exchange between conformational cluster substates giving rise to broad, weight-averaged resonances and (2) additional very slow exchange processes between configurational cluster substates in the β-domain. 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 temporarily breaking and reforming of the metal-thiolate bonds [90]. The existence of interchanging configurational cluster substates of comparable stability was demonstrated for inorganic adamantane-like metal–thiolate clusters with the general formula $[M_4(SPh)_{10}]^{2-}$ (M is Cd(II), Zn(II), Co(II), and Fe(II)) [91]. Studies toward obtaining the 3D NMR structure of mouse and human Cd_7MT-3 have also been undertaken [92, 93]. However, because of dynamic processes in the β-domain of Cd_7MT-3, only the 3D structure of the C-terminal α-domain (residues 32–68), containing an adamantane-like Cd_4Cys_{11} cluster, could be determined by NMR measurements. The structure of this domain reveals a peptide fold and cluster organization very similar to that found in mammalian Cd_7MT-1/MT-2, with the exception of an extended flexible loop encompassing the acidic hexapeptide insert (Fig. 3).

To account for slow dynamic events centered at the three-metal cluster of MT-3, a partial unfolding of the β-domain, whose kinetics could be determined by the *cis*/*trans* interconversion of Cys-Pro amide bonds in the C_6PCP_9 motif, has been suggested [94]. Support for this model came from extended X-ray absorption fine structure (EXAFS) studies of Zn_7MT-3 and its single $Zn_3\beta$-domain where, besides evidence for the tetrahedral tetrathiolate Zn(II) coordination, also indication of an unusual structure of the three-metal cluster was obtained. In both $Zn_3\beta$-domains a Zn···Zn backscattering at about 3.28 Å was detected (Fig. 4) [87]. Since this distance is inconsistent

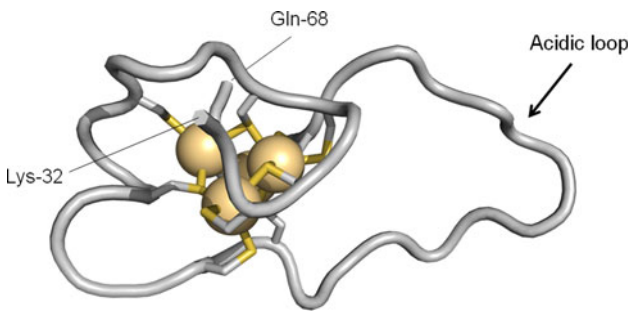

Fig. 3 The NMR solution structure of the α-domain of human $^{113}Cd_7$MT-3 [93]. The Cd(II) ions are shown as *light-orange spheres* connected to the protein backbone by cysteine thiolate ligands. The model was generated with PyMOL using the Protein Data Bank coordinates of 2F5H

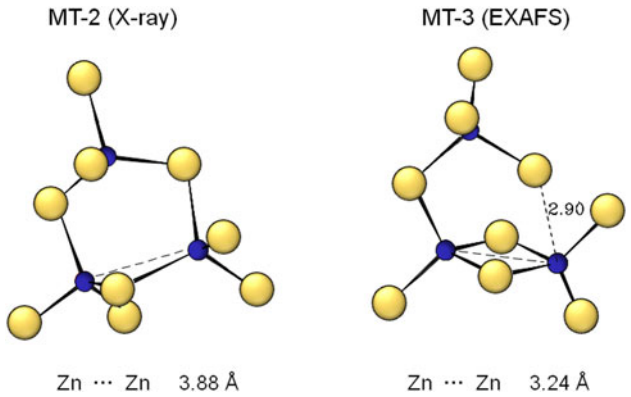

Fig. 4 Structural model of the $Zn_3(Cys)_9$ cluster in Zn_7MT-3 (*right*) and in comparison with the cyclohexane-like $Zn_3(Cys)_9$ cluster present in Zn_7MT-2 (*left*). Metal ions are shown as *blue spheres* connected to cysteine sulfur ligands (*yellow*). EXAFS: extended X-ray absorption fine structure. (Adapted with permission from [87])

with the cyclohexane-like Zn_3Cys_9 cluster structure present in the β-domain of mammalian MT-2, where a Zn···Zn distance of about 3.8 Å exists [56], it may reflect a predominant population of one cluster configuration under the conditions used (at 77 K).

Additional insights into the dynamics of the β-domain of Cd_7MT-3 were provided by molecular dynamics simulations. The simulation of the partial unfolding supported the proposed role of *cis*/*trans* interconversion of Cys-Pro amide bonds in the folding/unfolding process of the β-domain of Cd_7MT-3 [95]. In other molecular dynamics simulations, a correlation between the number of backbone amide hydrogen bonds to metal coordinated cysteines, NH–S^{γ} hydrogen bonds, in mammalian Cd_7MTs has been proposed to represent a controlling factor regulating the metal-thiolate cluster dynamics [96].

The C_6PCP_9 motif was the first segment found to be important for the extracellular growth inhibitory activity of Zn_7MT-3 [57, 94]. Mutational studies on biologically inactive Zn_7MT-1 revealed that besides the introduced C_6PCP_9 motif, also the unique Thr5 is required for the bioactivity and structure dynamics [97]. Further insights into the structural features underlying the bioactivity of Zn_7MT-3 came from a number of mutational studies which revealed that other amino acids in the β-domain along with domain–domain interactions, mediated partly through the acidic hexapeptide insert in the α-domain, are important (reviewed in [98]). Thus, although the mechanisms underlining these biological effects remain to be elucidated, the results obtained so far suggest that the structure of the β-domain of Zn_7MT-3 is subjected to a fine-tuning. Taken together, the findings of the structural and mutational studies led to the conclusion that both the specific structural features and the structure dynamics are necessary prerequisites for the extracellular biological activity of

Zn_7MT-3 [94, 97, 98]. The bioactivity in neuronal assays has been established for $Cu(I)_4Zn_{3-4}$MT-3 isolated from human [2] and bovine [99] brains and for recombinant human Zn_7MT-3 [98, 100]. However, comparative biological studies on well-defined metalloforms are currently lacking. Recently, two reviews focusing on various aspects of the structure and biology of MT-3 have been published [101, 102].

Structural features of Cu(I)-bound metallothionein-3

Whereas isolated mammalian MT-1 and MT-2 from livers of various species usually contain seven Zn(II), MT-3 isolated from human and bovine brains contains four Cu(I) and between three and four Zn(II). The isolated $Cu(I)_4Zn_{3-4}$MT-3 is a monomeric protein stable in air. EXAFS studies on $Cu(I)_4Zn_{3-4}$MT-3 revealed the presence of two homometallic clusters, a $Cu(I)_4$–thiolate cluster and a Zn_{3-4}–thiolate cluster [87]. The EXAFS data indicated that in contrast to tetrahedrally coordinated Zn(II) ions, Cu(I) ions are diagonally and/or trigonally coordinated by two or three cysteine ligands [87]. This observation, also seen for the Cu(I)–thiolate clusters in MT-1/MT-2, signifies that there are different coordination geometries for the binding of monovalent and divalent metal ions to MT-3. Therefore, to accommodate metal–thiolate clusters with different coordinating geometries, the structure should possess a high degree of flexibility.

Detailed information on the interaction of Cu(I) with MT-3 was forthcoming from spectroscopic studies of Cu(I) binding to the metal-free α-domain and β-domain and the full-length protein. The stepwise Cu(I) binding to both individual MT-3 domains showed that two well-defined Cu(I)–thiolate cluster forms are generated during this process. In the case of the β-domain (residues 1–32; Table 1), the successive formation of two cluster forms involving all nine cysteine ligands was observed, i.e., Cu_4S_9 and Cu_6S_9 clusters [88]. Similar studies on the α-domain (residues 32–68), containing 11 cysteine ligands, resulted in the formation of a Cu_4S_{8-9} cluster followed by a Cu_6S_{11} cluster [89]. In addition, the formation of the Cu_4S_9 cluster in both the single β-domain and full-length protein was cooperative [88, 103]. The cooperative formation of a stable folding intermediate containing a Cu_4S_9 cluster in the β-domain has also been shown by ESI–MS [104]. The major differences in the respective spectroscopic features between the $Cu(I)_4$ and $Cu(I)_6$ cluster forms were observed in the low-temperature Cu(I) luminescence spectra at 77 K [88, 89, 103]. Whereas the $Cu(I)_6$ cluster exhibited only a single emission band at 600 nm, in the case of the $Cu(I)_4$ clusters two emission bands at 420 and 610 nm were discerned. The presence of two emission bands in $Cu(I)_4$ clusters has been correlated with short intranuclear Cu···Cu distances (less than 2.8 Å), allowing metal–metal interactions due to a d^{10}–d^{10} orbital overlap. Accordingly, the high-energy emission band has been assigned to a ^{3}CC (cluster-centered) origin and that at low energy to triplet charge transfer [88, 89, 105]. The metal–metal interactions may contribute to the stability of the $Cu(I)_4$–thiolate cluster to oxidation in air. This is supported by the observation that the expansion of the metal core, generating a $Cu(I)_6$–thiolate cluster, results in increased Cu···Cu distances and susceptibility to oxidation [105]. Similarities between the luminescence spectra of isolated Cu_4,Zn_{3-4}MT-3 and the $Cu(I)_4$–thiolate cluster in the single β-domain suggested the presence of this cluster also in the isolated protein. Taken together the data indicated that the β-domain of MT-3 shows clear preference for binding of Cu(I) over Zn(II) ions.

The generation of a nativelike MT-3 structure through direct Cu(I) and Zn(II) incorporation into the recombinant apoprotein revealed monomeric Cu_4,Zn_4MT-3, in which, besides the $Cu(I)_4$ cluster in the β-domain, a Zn_4 cluster in the α-domain was present [103]. Whereas the $Cu(I)_4$ cluster in Cu_4,Zn_4MT-3 possesses the already mentioned stability to air oxygen, the Zn_4 cluster was found to be air-sensitive. Its oxidation resulted in disulfide formation and the release of one Zn(II), yielding Cu_4,Zn_3MT-3. This process could be prevented or reversed under reducing conditions [106].

Roles of metallothionein-3 in metal-linked neurodegenerative disorders

A predisposing risk factor associated with neurodegenerative diseases is age. In the normal brain a high concentration of essential transition metal ions such as zinc, copper, and iron ions is present. Dysregulated metal homeostasis, abnormal metal–protein interactions, and the associated oxidative stress, protein misfolding, and aggregation are critical common pathological hallmarks of the progression of several metal-linked neurodegenerative disorders [107, 108]. Insoluble protein deposits and diffusible oligomers are composed of individual amyloidogenic proteins or peptides such as the Aβ peptides, a major component of extracellular amyloid plaques in Alzheimer disease, prion protein in prion deposits typical of Creutzfeldt–Jakob disease, α-synuclein (α-Syn) in intracellular Lewy bodies in Parkinson disease, superoxide dismutase (SOD-1) aggregates in amyotrophic lateral sclerosis, and Huntington inclusions in cases of Huntington disease. In these neurodegenerative diseases, the expression of MT-3 has been found to be downregulated or altered [109–111] and changes in normal homeostasis of essential transition metals such as zinc and copper have been implicated as possible etiological factors [108, 112]. In contrast to redox-inert zinc, redox-active copper aberrantly bound to

amyloidogenic proteins can react with molecular oxygen, resulting in the production of ROS through Fenton and Haber–Weiss reactions [113]. That MT-3 may play an important role in the progression of these diseases has been reviewed [111].

A protective role of extracellular Zn_7MT-3 from Cu(II) toxicity has been suggested on the basis of investigations of its reactivity toward free Cu(II) ions [114]. The results showed that Zn_7MT-3 through Cu(II) reduction to Cu(I) by thiolate ligands and binding to the protein, forming an air-stable $Cu(I)_4Zn_4$MT-3 species, efficiently scavenges and redox-silences the free Cu(II) ions. In this reaction, a $Cu(I)_4$–thiolate cluster is formed cooperatively in the β-domain of the protein concomitant with two intramolecular disulfide bonds and the release of three Zn(II) ions [114]. Since four Zn(II) ions remained bound, the presence of an intact Zn_4 cluster in the α-domain was suggested. The formation of $Cu(I)_4Zn_4$MT-3 completely quenched the ascorbate-driven and Cu(II)-catalyzed production of ROS [114]. Taken together the findings indicate that two types of air-stable $Cu(I)_4$–thiolate clusters can be formed in the more reactive β-domain of MT-3, i.e., a fully reduced $Cu(I)_4S_9$ cluster and a $Cu(I)_4S_{5+x}$ cluster. In the latter, by analogy with inorganic model complexes, the participation of a disulfide bridge sulfur (indicated by x) besides thiolates in the Cu(I) coordination cannot be excluded.

Studies aimed at understanding the protective effect of human Zn_7MT-3 against $A\beta_{1-40}$ toxicity showed that the protein can efficiently remove copper not only from soluble $A\beta_{1-40}$–Cu(II) oligomers, but also from insoluble aggregates. In this process, Cu(II) is reduced by protein thiolates, forming the stable $Cu(I)_4Zn_4$MT-3 species described above and the non-redox-active $A\beta_{1-40}$–Zn(II). This metal swap completely quenches the ROS production mediated by Cu(II) bound to $A\beta_{1-40}$ and occurs not only in vitro, but also in human neuroblastoma cell culture, whereby the toxic effect of $A\beta_{1-40}$–Cu(II) is abolished [115]. In recent studies, the protective effect of human Zn_7MT-2A against $A\beta_{1-40}$–Cu(II) toxicity was also investigated and compared with that of Zn_7MT-3 [116]. In neuronal cell culture, a similar protective effect has been shown. The results of the reaction between $A\beta_{1-40}$–Cu(II) and Zn_7MT-2A have been discussed together with the chemical data on the fully reduced $Cu(I)_{10}$MT-2A and $Cu(I)_{12}$MT-3 species, generated in the reaction of apoMT-2A or apoMT-3 with the Cu(I)-DTT complex under strictly reducing conditions. It has been suggested, moreover, that the increased affinity of MT-2A for Cu(I) over MT-3 makes MT-2A a better protecting agent [116]. However, as the reduction of Cu(II) in $A\beta_{1-40}$–Cu(II) to Cu(I) is accomplished by the thiolate ligands of both MTs, the formation of fully reduced Cu(I)MT-2A/MT-3 metalloforms in the oxidizing extracellular environment is highly unlikely. That under these conditions the reaction between $A\beta_{1-40}$–Cu(II) and Zn_7MT-2A also leads to $Cu(I)_4Zn_4$MT-2A, in which a $Cu(I)_4$–thiolate cluster and two disulfide bonds are present, has been revealed (G. Meloni, C.L. Seeland, and M. Vašák, unpublished). Note also that the implicated direct metal swap between Cu(II), coordinated by nitrogen and oxygen ligands in $A\beta_{1-40}$–Cu(II), and Zn(II), coordinated by thiolate ligands in Zn_7MT-2A, without preceding Cu(II) reduction cannot occur. Consequently, the differences in Cu(I) affinity for MTs are not essential for the protective effect, but rather the long-term stability of partially oxidized Cu(I),ZnMTs formed upon Cu(II) reduction and removal from $A\beta_{1-40}$–Cu(II) in the oxidizing extracellular space are essential. The ensuing redox cycling of copper in oxygen-sensitive Cu(I),ZnMTs can change their properties from antioxidant to prooxidant. In this context it may be noted that although the MT-1/MT-2 isoforms have been found to be significantly upregulated in regions of $A\beta$ plaques in the Alzheimer disease brain [117–120], the presence of substantial concentrations of Cu(II) in these plaques (0.3 mM) has been shown [108, 121]. Clearly, more studies regarding the stability of brain MTs formed in the reaction between Zn_7MTs and different concentrations of Cu(II) to molecular oxygen are needed.

Besides Alzheimer disease, a protective effect of MT-3 has also been considered in other metal-linked neurodegenerative diseases such as Parkinson disease and prion diseases [111]. Studies conducted on hemiparkinsonian rats suggest that the free-radical scavenging potency, including that of MT-3, is reduced in the Parkinson disease brain [111]. In Parkinson disease, the fibrillation and aggregation of α-Syn is a key process in the formation of intracellular inclusions, Lewy bodies, in neurons of substantia nigra pars compacta [122]. Aberrant tight binding of one Cu(II) to α-Syn and associated oxidative stress appears to contribute to the degeneration of dopaminergic neurons through the abnormal aggregation of this protein [123–125]. In prion diseases, transmitted by proteinaceous infective agents (prions) [126], the transition from natively folded prion protein (PrP^C) to misfolded prion protein (PrP^{Sc}) is a crucial pathogenic event [107, 127]. The mainly disordered part of the PrP^C structure can bind up to six Cu(II) ions [107]. Also, in this case oxidative stress, associated with the copper-catalyzed transformations of prion protein, plays an important role in the disease progression. Recent in vitro studies into the role of Zn_7MT-3 in Parkinson disease and prion diseases have shown that Zn_7MT-3, through Cu(II) removal from α-Syn and prion protein and the formation of air-stable $Cu(I)_4Zn_4$MT-3 species, efficiently prevents the deleterious redox activity of these proteins [128]. In view of widely different Cu(II) binding motifs in $A\beta$, α-Syn, and prion proteins, a general protective role of Zn_7MT-3 against Cu(II) toxicity in the brain can be envisaged.

Metallothionein-4

The last member of the mammalian MT gene family *MT-4* is located about 20 kb upstream from the 5′ of the *MT-3* gene in both the mouse and the human genome [3]. MT-4 consists of 62 amino acids, with an insert of glutamate in position 5 relative to the classical MT-1 and MT-2 proteins (Table 1). Murine MT-4 messenger RNA appears to be present exclusively in cornified and stratified squamous epithelia. Many of these epithelia develop parakeratosis during zinc deficiency in rats. Differentiation of stratified epithelium involves movement of keratinocytes out of the basal, proliferative layer into the overlaying region where synthesis of unique cytoskeletal proteins begins. In situ hybridization revealed intense labeling of MT-4 messenger RNA in the differentiating spinous layer of cornified epithelia, whereas MT-1 was expressed predominantly in the basal, proliferative layer. Thus, a switch in MT isoform synthesis occurs during differentiation. The MT-4 derived from tongue epithelium contained both zinc and copper (ratio 2.6:1) [3]. From molecular and cell biology studies, involvement of MT-4 in regulating zinc metabolism during the differentiation of stratified epithelia has been suggested [3]. Molecular biology and expression profile studies of MT-4 in mammalian maternal decidua [129] and during epithelia development and physiology [3, 130] revealed that the *MT-4* gene is subject to a strict developmental regulation.

The function of MT-4 in handling divalent Zn(II) or monovalent Cu(I) metal ions has been inferred from its structural properties. The organization, stability, and assembly of divalent metal ions in mammalian MT-4 have been investigated using the Cd(II) and Co(II) metalloforms of the protein and have been compared with the well-characterized mammalian Cd_7MT-2 [131]. Both ^{113}Cd NMR studies of reconstituted $^{113}Cd_7$MT-4 and spectroscopic characterization of Co_7MT-4 revealed that, similarly to the classical MT-1 and MT-2 proteins, the seven divalent metal ions are organized into two independent Cd_4Cys_{11} and Cd_3Cys_9 clusters, with each metal ion tetrahedrally coordinated by terminal and μ-bridging cysteine ligands [131]. The cluster formation in Cd_7MT-4 was cooperative and sequential, with the Cd_4Cys_{11} cluster in the α-domain being formed first. In addition, the metal–thiolate clusters in MT-4 appear more stable to demetallation by EDTA than those of MT-1 [132]. The decreased ligand substitution reactivity of EDTA observed with Cd_7MT-4 presumably reflects marked differences in the cluster geometry in Cd_7MT-4 compared with Cd_7MT-1/MT-2 [131].

Insights into the Cu(I) binding specificity of MT-4 have been obtained from the characterization of metalloforms upon heterologous expression of MT-4 and its individual domains in *Escherichia coli* culture using zinc-, cadmium-, or copper-supplemented media and their comparison with the well-characterized metalloforms of MT-1 [133]. Spectroscopic and ESI–MS analyses of the metal composition of the purified full-length protein and the individual α- and β-domains revealed that compared with MT-1, the MT-4 isoform shows an increased preference for monovalent copper ions over divalent zinc or cadmium ions, properties reflected in the formation of mixed M^{II} (M is Zn, Cd) and Cu(I) species [133]. On the basis of these results and the in silico protein sequence analyses, it has been concluded that MT-4 differentiated to a MT with a "copper thionein" character rather than to a MT with a "zinc thionein" character. It has been suggested, moreover, that the dissimilarities in non-cysteine residues between MT-4 and MT-1 are responsible for the differences in metal binding behavior. Furthermore, the diminished Cd(II) binding ability of Cd_4-αMT-4 versus Cd_4-αMT-1 has been attributed to its high proline content, in agreement with the association of Cys-Pro motifs to structural perturbations in MT-3 [97]. Overall, the structural studies support the role of MT-4 in both zinc and copper metabolism in epithelia. Nevertheless, more work regarding the 3D structure of MT-4 and its physiological role are needed.

In more recent studies, the presence of about 14 and 3 mol of sulfide (S^{2-}) ligands in recombinant full-length CdMT-4 and CdMT-1, respectively, has been reported [134, 135]. This finding led to the reinterpretation of the all assumed heterometallic Zn,Cd-βMT-4 species in the previous heterologous expression of MT-4 [133] as Cd-βMT-4 complexes that include sulfide ligands. However, although in these studies convincing analytical evidence for the presence of sulfide in these and other recombinant preparations of mammalian MTs was provided, in the analyses for MT isoforms present in the cytosol of mammalian cells and tissues by the combination of various analytical techniques, including capillary electrophoresis (CE) inductively coupled plasma MS and CE–ESI–MS, mass peaks of fully metal occupied MT isoforms without sulfide have been reported [136, 137]. Since in these studies MT isoforms containing sulfide may have escaped detection owing to their substantially increased molecular masses, more studies are needed to clarify the presence of sulfide in mammalian MTs.

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