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The Metallothionein/Thionein System: An Oxidoreductive Metabolic Zinc Link

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Metallothioneins (MTs) were discovered more than 50 years ago and identified as low-molecular weight, sulfhydryl-rich proteins that were subsequently found to bind zinc predominantly. The binding of seemingly redox inactive zinc ions allows MT to play a central role in oxidoreductive cellular metabolism, cellular zinc distribution and homeostasis. In this interpretive study, we discuss the interaction of MT with physiologically relevant molecules and its effect on zinc–thiolate bonds. These interactions are linked to recent progress in the functional role of MT in cellular zinc transport, energy production, and protection of the organism against oxidative stress and neurodegenerative diseases.

Introduction

In 1869, Jules Raulin, a student of Pasteur, discovered the presence of zinc in *Aspergillus niger*, the common bread mold.[1] Due to the metal's unique properties, distribution, and concentration in tissues, zinc's biological roles were slow to be established, appreciated, and accepted. The lack of color of zinc-based compounds as compared with compounds of iron and copper further delayed its detection for half a century. It is difficult, now, to appreciate that the very detection of zinc in biological matter presented a seemingly insurmountable obstacle only a very short time ago.

In the course of the last century, the advent of new technologies (for example, atomic absorption and other spectrometries), has reduced the detection limits of zinc from *milli*grams to *femto*grams.[2–4] These technological advancements have made zinc metabolism more accessible in the time frame of biological events. More recently, the development of fluorescence-based sensors and biosensors has further clarified its biological functions.[5,6]

Zinc is now universally recognized to be essential for growth at all levels and is indispensable for transcription and translation of the genetic message, cell division, differentiation and morphogenesis.[3,7] Additionally, zinc propagates neural activity, rendering the physiology and pathology of neural events—their recognition, prevention, and therapy—accessible to study.[8,9] Zinc storage disorders, comparable to or associated with those of copper and iron, for example, are unknown. This suggests the existence of efficient systems that direct zinc to places when and where it is needed.[10]

The number and types of zinc-containing and/or zinc-dependent proteins that have been recognized are startling, but their recognition now goes hand in hand with studies of their mechanisms of action. The catalytic properties of zinc were established after its discovery in carbonic anhydrase.[3] The study of this and other zinc enzymes such as carboxypeptidases A and B, alcohol dehydrogenases, alkaline phosphatases, and thermolysin, formed the basis of zinc metalloenzymology.[11] The functions of zinc in enzymes were then designated as catalytic, coactive (or cocatalytic) and structural.[3]

These realizations were pivotal. The apparent resistance of zinc to oxidoreduction accounts both for its biological potential and versatility. The metal is amphoteric and its coordination sphere varies from two to eight and is an important means for the translation of chemical structures into multiple and varied biological messages.[3,12]

One other major characteristic of zinc chemistry is well known but not always cited in context: zinc and sulfur are complementary. The history of zinc in biology does not stress this rule of thumb, taught by geology. Subsequent discoveries of zinc "fingers," "twists," "rings," and "clusters" have immensely enlarged the scope of its biological and therapeutic potentials.[3,13] The mammalian zinc "cluster" protein metallothionein (MT) has been found to play critical roles for zinc, both in homeostasis and distribution, as well as in neural and metabolic networks.[14,15]

Metallothionein

MT was discovered in 1957 and has been studied intensively since then, but its metabolic roles have remained elusive.[16] Uchida was the first to suggest that it might be a neuronal growth inhibitor (MT-3).[17] Recently, MT was also found to act as a reducing agent for methionine sulfoxide reductase.[18]

The composition and structure of MT are fascinating. Its molecular weight, ~7 kDa, is remarkably low. Four distinct mam-

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malian MT isoforms, designated MT-1 through MT-4, have been detected and investigated so far.[19] MT-1 and MT-2 are found in all organs, whereas MT-3 is expressed mainly in the central nervous system, and MT-4 is most abundant in stratified tissues. All four isoforms contain 20 conserved Cys, and at least five conserved Lys residues (MT-1 and MT-2 have eight conserved Lys). His, Phe, Tyr, or Trp residues are completely absent in all of them (Figure 1). Consequently, MT does not absorb radiation at or near 280 nm as is characteristic of conventional proteins, and thereby escapes the routine methods of protein detection.

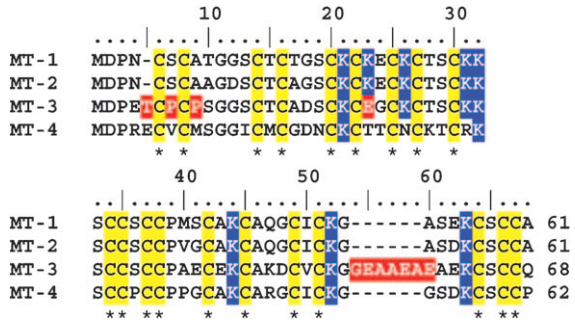


Figure 1. Sequence alignments of the four mammalian forms of MT (*H. sapiens* MT1A, MT2A, MT3 and MT4) with the β-domain on top and the α-domain below. Conserved cysteines (*) and lysines are highlighted in yellow and blue respectively. Key differences in the MT-3 sequence are highlighted in red.

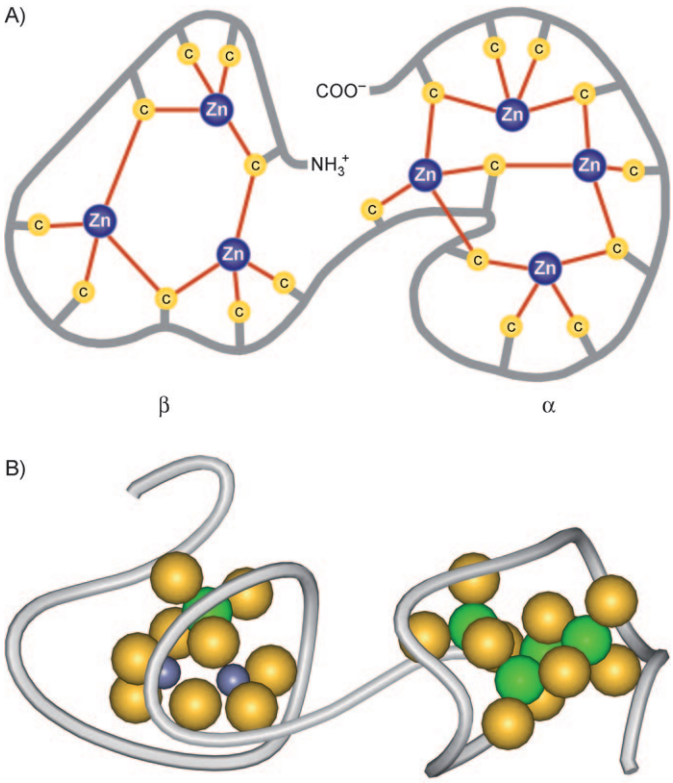


Figure 2. A) The α- and β-zinc-binding domains of MT and B) the structure of rat Cd_5Zn_2MT-2 (PDB ID: 4MT2). Four cadmium atoms are bound in the α-domain with one cadmium and two zinc atoms bound in the β-domain: Cd (green) with Zn (blue) and S (gold).

While MT is mainly found in association with zinc, it also binds a wide range of other metal ions, including Cd^{II}, Hg^{II}, Cu^{I}, Cu^{II}, Ag^{I}, Au^{I}, Bi^{III}, As^{III}, Co^{II}, Fe^{II}, Pb^{II}, Pt^{II} and Tc^{IV}.[20] X-ray crystallography and NMR spectroscopy show that the protein is arranged in two domains, (α and β) with their constituent cysteines in "cluster" formations, enveloping the metal atoms.[21,22]

No such characteristic structure had been previously recognized in nature, rendering the system truly unique to both biology and chemistry. Five cysteines bridge the metal atoms in the C terminal α-domain, where four zinc atoms are bound to eleven cysteines. In contrast, the N terminal β-domain is formed by three zinc atoms and nine cysteines (Figure 2). These arrangements (28 intramolecular zinc–sulfur bonds) account for the extremely tight zinc binding in both clusters and for the zinc-donating properties of MT.[23,24] Recent work has suggested that inorganic sulfide ligands may be present in the clusters.[25]

In the α- and β-clusters, the affinities for metal binding, zinc transfer rates, and chemical reactivity differ significantly. The isolated β-cluster reacts faster with oxidizing agents and is a better zinc donor towards zinc-depleted sorbitol dehydrogenase than the isolated α-cluster, while the reverse is observed when a chelating agent is the zinc acceptor. Holo-MT is stabilized with regard to the individual domains. Their cumulative properties neither describe MT structurally nor functionally. The two-domain structure of the holo protein is important for its interaction with ligands and for the control of its reactivity and overall conformation.[23]

There are two insertions in the amino acid sequence of human MT-3 as compared to MT-1 and MT-2; these include a six amino acid insertion in the α-domain and a single threonine (Thr-5) insertion in the β-domain. MT-4 has a glutamate insertion in the β-domain at the 5-position. These differences, and others in the amino acid sequence, may account for any functional differences between the different MT isoforms.

Zinc Transfer

In addition to the peculiarities of zinc in MT and unlike transition metals in biology, zinc is mobilized and delivered throughout the cell by processes that do not alter its valence. The concentration of "free" zinc in the cell has been shown to be very low; thus, its movement must be regulated tightly.[4] Several membrane-bound zinc transporter proteins have been identified and characterized. These are arranged into two families: ZnT proteins for cellular zinc removal and Zip proteins for zinc uptake.[10] The crystal structure of a bacterial homologue of the cation diffusion facilitator ZnT proteins (Yiip from *E. coli*) has been reported recently.[26] A postulated mechanism for intracellular zinc transport may have MT as its cornerstone. We have proposed that certain biological zinc/thiolate "cluster" motifs can bind zinc tightly while retaining reactivity as zinc donors. (Examples of this are the four-zinc and three-zinc clusters in the α- and β-domains of MT and the two-zinc cluster in the Gal4 protein, which is a transcription factor).[27,28]

Towards this end, exchange of stable zinc with ^{65}Zn has served to probe the coordination dynamics of zinc "clusters." When mixed, MT-1 and -2 rapidly exchange zinc with each other. Rapid chromatographic separation followed by radioactive detection shows two distinct phases ($k_{fast} \approx 5000\ min^{-1}\ M^{-1}$ and $k_{slow} \approx 200\ min^{-1}\ M^{-1}$, at pH 8.6, 25 °C).[29] These rates are thought to reflect the zinc exchange between the β- and α-domains, respectively.

Zinc exchange ($k \approx 800\ min^{-1}\ M^{-1}$, pH 8.6, 25 °C) was also observed between MT-2 and the Gal4 protein, which contains a two-zinc cluster. This represents the first intermolecular zinc exchange reported among heterologous proteins, which has now been well documented. MT also transfers zinc to alkaline phosphatase, carboxypeptidase A, sorbitol dehydrogenase and glycerol phosphate dehydrogenase.[15,28,30] Zinc exchange between MT and zinc "finger" transcription factors has also been observed (for example, TFIIIA and SP1). This zinc transfer has been speculated to regulate gene expression through activation or inhibition of DNA binding.[15,28] In fact, zinc can be shuttled in both directions—from MT to zinc apo enzymes and from zinc enzymes to metal-free metallothionein (thionein, T).

Zinc transfer from MT to mitochondrial aconitase has also been demonstrated in cell tissue. Heart extracts from MT-null mice were incubated with ^{65}Zn-MT or $^{65}ZnCl_2$ to identify differentially labeled proteins.[31] The zinc exchange was shown to be due to a direct interaction between MT and aconitase. Further evidence for a direct interaction between MT and apo zinc-binding proteins was observed when these peptides were separated from MT by a membrane through which only zinc ions can diffuse. Zinc exchange was not detectable in the membrane separated system, compared with the rapid exchange when the apoprotein and MT are mixed.[28] These important observations imply that bound zinc can only be delivered when a direct interaction between MT and zinc acceptors occurs (that is, MT can serve as a zinc specific chaperone).

While zinc is often required for enzymatic activity, it can also *inhibit* some enzymes; they include caspase-3, fructose 1,6-diphosphatase, glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase, tyrosine phosphatases, and yeast enolase among others. The addition of metal-free metallothionein can restore their enzymatic activity, thereby acting as a chelating agent which removes and sequesters zinc from the inhibited enzymes.[32]

Redox Activity of MT

While molecular zinc is redox inert, the cysteine sulfur ligands of MT can be oxidized and reduced concomitantly with the release and binding of zinc and the formation and cleavage of disulfide bonds. This oxidoreductive mechanism confers redox activity on MT, with the redox chemistry originating from the metal and its coordination environment. Hence, the coordination of zinc can become part of the redox environment of the cell. Since the reduction potential of MT remains sufficiently low (less than −366 mV), a number of physiological oxidants, including disulfides and selenium compounds, can effect MT oxidation.[15,28,33]

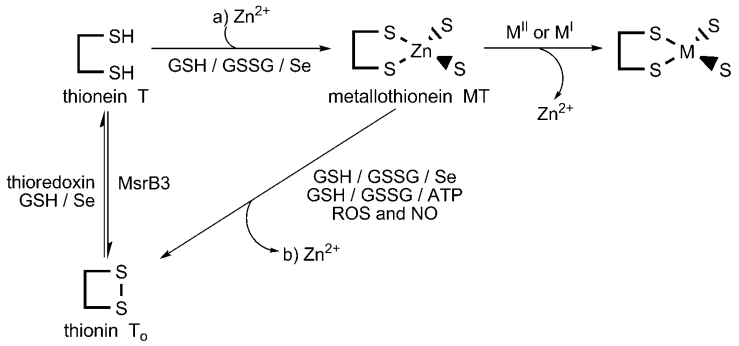
MT interacts with glutathione disulfide (GSSG) to release zinc. The solvent-accessible zinc-bound thiolates of each domain participate in a thiol/disulfide interchange with GSSG, in which the initial interaction is the rate-limiting step; this causes the clusters to collapse and release their zinc. This mechanism was previously unknown and may play a significant role in cellular zinc distribution. Since GSSG and its reduced form, glutathione (GSH), represent about 90% of cellular nonprotein sulfur, the GSH/GSSG couple is thus a major determinant of the cellular redox state. With their inherent redox behavior and zinc complementarity, sulfur donor ligands have been depicted as eager acceptors/receptors of zinc from MT.

In zinc transfer reactions, MT does not release all of the seven zinc atoms. At least one of them is more likely to transfer than the balance. Full reactivation of metal-free sorbitol dehydrogenase requires a 1:1 molar ratio of MT; this indicates that only one of the seven MT zinc atoms is transferred. Both reduced GSH and GSSG modulate zinc release and transfer.[34] GSSG oxidizes MT, enhancing the release and transfer of zinc from MT three-fold, whereas GSH mediates zinc transfer from enzymes to T. The GSSG concentration is the major determinant of the rate. GSH has a dual function. In the absence of GSSG, it inhibits zinc transfer from MT; this indicates that MT is in a latent state when the cellular concentrations of GSH are relatively high. However, when both GSH and GSSG are present, zinc release is increased ten-fold and four zinc atoms can be transferred.[34] Therefore, both GSH and GSSG are critical modulators of the rates of zinc transfer and the ultimate number of zinc atoms transferred. The zinc/thiolate interactions and the structure of MT are both crucial in these interactions, as well as in the regulation of zinc transfer (Scheme 1).

The stability constants (K) of most zinc enzymes are three orders of magnitude lower than the measured overall stability constant of MT ($K \sim 10^{12}$-$10^{13}\ M^{-1}$); this renders zinc transfer from MT thermodynamically unfavorable. However, the redox mechanism at the MT zinc-thiolate cluster can overcome this thermodynamic barrier and release additional zinc on demand.

All seven zinc atoms in MT seem to have similar coordination environments, yet three distinct classes of zinc sites have been observed that have stability constants that span over four orders of magnitude. The first class of sites (Zn_{1-4}) is bound more strongly than the second (Zn_5 and Zn_6) However, the stability constant of one zinc atom, (Zn_7), is significantly lower ($K_d = 2.1 \times 10^8\ M^{-1}$) than the other six.[24] This thermodynamically enables the transfer of one zinc atom from MT to many other zinc binding proteins.

Under normal in vivo conditions, MT forms disulfide bonds after oxidative zinc release, as indicated by differential alkylation of its cysteine residues. The number of disulfide bonds increases under conditions of oxidative stress.[35] Structurally, the availability of 20 cysteines in MT would favor intramolecular disulfide bond formation, but the high concentrations of MT in vitro allow for the formation of dimers under oxidative conditions.[22] The exact mechanism of disulfide bond formation is unknown, although it has been found to be intra- rather than intermolecular disulfide bonds located in both the α- and β-domains have been observed.[22] Once formed, the disulfide



Scheme 1. Metallothionein redox cycle and its link to the function of MT in zinc homeostasis. Zinc transfers are modulated by the GSH/GSSG ratio, selenium compounds, and ATP. a) Removal of "free" Zn or Zn from enzymes (for instance caspases, tyrosine phosphatase). b) Zn activation of proteins and transcription factors (for instance sorbitol dehydrogenase and MTF-1) and Zn inhibition (for example respiratory chain complexes).

bonds can be reduced by GSH but not by chemical reducing agents such as 2-mercaptoethanol and dithiothreitol. However, even at high concentrations of GSH or GSSG, the redox processes are relatively slow.

Involvement of Selenium

Selenium containing peptides have been inferred to play an important role in MT metabolism. The GSH/GSSG redox pair efficiently couples with the MT/T system in the presence of selenocystamine, which can form a catalytic selenol(ate). This has been suggested to be a very effective means of modulating oxidoreduction under the reducing conditions in the cytosol. Thus, the binding and release of zinc from zinc-thiolate coordination sites could be linked to redox catalysis by selenium compounds, and thereby to changes in the GSH redox state and to the availability of either a zinc donor or acceptor (Scheme 1).[15,28,36]

Selenium is biologically essential and is an element whose derivatives protect cells against oxidative stress; it also has exhibited anti-neoplastic properties.[37] The interaction of selenium (and tellurium) compounds with MT and GSH reveals a potential role for these in the development of novel therapeutic agents with zinc-releasing and antioxidant properties.[38,39] Specifically, selenium may be incorporated into proteins in the form of selenocysteine (Sec) and nonspecifically as selenomethionine.[40] It may therefore be possible to design and synthesize therapeutic agents and enzymes in which sulfur atoms are replaced by Se.

In vivo, zinc always seems to associate with other cellular ligands and its movement is tightly regulated, while the amount of "free" zinc remains very limited.[4] Chelating agents such as tris buffer, citrate, or GSH mediate its transfer from zinc enzymes to T.[41] The concentration of "free" zinc and the redox state of the cell ultimately determine the overall distribution of zinc from the MT/T system, but the number and identity of its components has yet to be determined for most specific instances. In vivo, such a system might keep the cellular concentrations of "free" zinc very low, and by acting as a temporary cellular reservoir, release zinc in a process that is dynamically controlled. Therefore, a change in the redox state of the cell could serve as a driving force and signal for zinc distribution from MT, as controlled by the zinc/thiolate cluster domains and catalyzed by selenium derivatives (Scheme 1).

MT and Respiration

ATP and GTP form 1:1 complexes with MT-2 [K_d-(ATP) = 176 ± 33 μM, pH 7.4].[42] This interaction also enhances the transfer of zinc to zinc-depleted sorbitol dehydrogenase. Thus ATP (but not the corresponding di- or monophosphates and pyrimidine nucleotides) modulates zinc exchange from MT in a manner similar to that of GSH/GSSG. Carbamoylation of the lysines in MT totally abolishes ATP binding, suggesting that these highly conserved residues are part of the ATP binding site. Subsequent work by others could find no evidence of ATP binding to MT.[43] However, NMR studies and scanning tunneling microscopy provided further evidence for ATP binding and showed that chloride ions could potentially interfere with ATP binding.[44] The interaction of ATP also affects the N- and C-terminal amino acids of MT, enhancing the rate of thiol-disulfide interchange with Ellman's reagent [5,5-dithiobis-(*Z*-nitro benzoic acid)], implying a change in the conformation of MT.[44]

Scanning tunneling microscopy demonstrates that, in the presence of ATP, the linear MT molecule bends by about 20° at the central hinge between the α- and β-domains.[44] GSH inhibits ATP binding while GSSG enhances it. As a consequence, the amount of ATP bound to MT in the cell is a function of the GSH/GSSG couple. These interactions may be important in activating each cluster for zinc transfer in vivo.

The inhibition of oxidative phosphorylation by zinc has been known for many years. All four complexes of the respiratory chain are affected but only complexes I and III are inhibited with high affinity.[45] There are two zinc binding sites in the structure of complex III.[46] MT has also been proven to inhibit oxygen consumption of intact mitochondria.[47] MT-null mice (with MT-1 and -2 genes disrupted) develop a mild form of obesity.[48] The incubation of mitochondria with physiological, (micromolar) concentrations of MT results in the import of MT into the mitochondria (the lysines of MT have been postulated to be important in this transportation).

MT localizes in the mitochondrial inner membrane space of the liver but not of the heart. The mitochondria and the electron transport chain are the primary sites of inhibition.[49] Free zinc inhibits respiration at concentrations commensurate with the zinc content of the MT or the isolated β-domain. Consequently, MT could make zinc available for the inhibition of the electron transport chain in mitochondria. Alternatively, respiration can be restored by the addition of T.[49] These observations have resulted in speculation that zinc might serve as a pacemaker of energy production, a reaction that would be controlled strictly by the redox-linked MT/T system (Scheme 1).[28,50]

MT and Oxidative Stress

Reactive oxygen and nitrogen species (ROS and RNS) are known to cause oxidative damage, which implicates them in major neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS; motor neuron disease), Parkinson's, Creutzfeldt-Jakob and Alzheimer's diseases, as well as cardiovascular disorders, respiratory distress syndrome, rheumatoid arthritis, atherosclerosis, diabetes, essential hypertension, muscular dystrophy, cystic fibrosis, ulcerative colitis and aging.[51,52]

The generation of ROS and RNS is inherently linked with metabolism and respiration. Their metabolism involves many enzymes, including catalase, superoxide dismutase, reductase enzymes and peptides such as GSH.[53] In vitro, MT interacts with numerous ROS (for example, superoxide, hydrogen peroxide, hydroxy radicals, nitric oxide, and peroxynitrite) which is followed by the release of zinc.[36] In fact, MT is more effective in quenching radicals than GSH. ROS and oxidative stress also increase the expression of MT-1 and MT-2 by way of the metal response element-binding transcription factor-1 (MTF-1, a zinc binding protein), which is the predominant regulatory protein mediating MT induction.[54] Studies with both MT-overexpressing and MT-null mice have further demonstrated that MT can also protect against oxidative conditions.[36]

The induction of MT expression by the binding of zinc to MTF-1 ($K_d < 90$ μM) has been demonstrated in vivo both under conditions of oxidative stress and in the presence of toxic metals.[55,56] Several other zinc responsive transcription factors (for example, the Zur and ZntR metalloregulatory proteins, which control the expression of zinc uptake and export genes) display femtomolar levels of zinc sensitivity.[4]

Recently, a heat stable protein, obtained from bovine liver, was identified as a zinc-containing MT which was found to act as a reducing agent for certain forms of zinc-containing methionine sulfoxide reductase (MsrB2 and MsrB3).[18] These MsrB proteins reduce methionine sulfoxide, reversing the oxidation of methionine by ROS. The expected physiological reducing agent thioredoxin turned out not to be active for all of the families of Msr enzymes. It seems possible that T could function as a reducing system for human MsrB3 by forming intramolecular disulfide bonds. Thioredoxin could then reduce these disulfide bonds in oxidized T (thionin, T_o) to regenerate T. Subsequent work has shown that selenocystamine, by catalyzing the exchange of zinc from MT/T, could also reduce the methionine sulfoxide reductase enzymes using thioredoxin reductase or T as the reducing agent.[57]

The roles of MT and zinc distribution in respiration and the protection against oxidative damage are potentially linked. The release of zinc from MT under conditions of oxidative stress and the interaction of MT with physiologically relevant oxidants and reducing agents mentioned earlier highlight the importance of MT in protecting the cell against oxidative damage (Scheme 1). The aforementioned catalytic role of selenium derivatives in controlling the release and binding of zinc may be crucial for these mechanisms.[15,28,50]

MT and the Central Nervous System

Zinc acts as a signaling agent in synaptic vesicles.[9] Along with glutamate, zinc is stored selectively in and released from the presynaptic vesicles of neurons in the cerebral cortex and can then enter the postsynaptic neurons through a variety of zinc-permeable gated channels. The metal seems to modulate the overall excitability of the brain through its effects on receptors and ion channels, thereby playing an important role in synaptic plasticity. However, excess zinc is toxic to neurons and various mechanisms have been proposed to explain this toxicity.[9,58] MT is involved in a synergistic relationship between zinc ions and nitric oxide. The latter is also a modulator of neurotransmitter release by activating the guanylate cyclase system.[59] Reaction of NO with MT causes zinc release and formation of disulfide bonds. Nitric oxide can be oxidized and reduced while zinc ions are redox insensitive. This reactivity suggests that through its zinc/thiolate bonds, MT may be involved in the transduction of a NO signal into a zinc signal.[8]

When compared with MT-1 and MT-2, the behavior of MT-3 (a growth inhibitory factor expressed mainly in the central nervous system) is more complex.[60] This suggests a different role for this MT isoform. Isolation of MT-3 yields a Cu_4Zn_{3-4} species, in which the copper species are said to be in the β-domain and zinc species in the α-domain.[14,61] Growth inhibitory activity in neuronal cell cultures has been found for the Zn_7, the Cu_4Zn_3 forms of MT-3 and its isolated N-terminal $Zn_3\beta$ domain.[61,62] MT-3, but not MT-1 or MT-2, was also found to protect neurons from the toxic effects of amyloid β-peptides.[63] The Thr5 insert, Pro7, Pro9 and Gln23 in the β-domain are essential for the specific activity of the MT-3 isoform when compared to MT-1 and MT-2 (Figure 1).[22,64] The hexapeptide insert in the α-domain has been implicated in a looser binding of zinc in this cluster, consistent with the observation of a redox labile zinc in the α-domain.[19,65] In contrast to MT-1 and MT-2, only the α-domain of MT-3 has been characterized structurally in detail.[66] Attempts to define the structure of the β-domain by way of NMR techniques have been hampered by its rapid internal dynamics. These rapid dynamics have been postulated to be important in determining the different properties of MT-3 as compared with the other isoforms.

Both copper and zinc have been implicated in neurodegenerative diseases (for example, amyotrophic lateral sclerosis, Alzheimer's, Parkinson's and Creutzfeldt–Jakob diseases).[67,68] In Alzheimer's disease, copper binds to the β-peptide, and in Parkinson's to the α-synuclein protein.[69] Copper and zinc are linked to free radical damage implicated in the above neurodegenerative diseases but are also involved in protecting against ROS through the Cu- and Zn-superoxide dismutases.[70] Genetic variants of the superoxide dismutase enzymes promote protein aggregation, which leads to amyotrophic lateral sclerosis.[71] A zinc-dependent peptidasome has been shown to degrade the amyloid β-peptide, which is associated with Alzheimer's disease.[72] Neuroinflammation associated with the above disorders up-regulates MT-1 and MT-2, while some investigators report a down-regulation of MT-3 instead.[14,73,74]

Cadmium bound to MT-3 can be replaced by reaction with Cu^{II}.[75] Recent work suggests that the interaction of Zn_7MT-3 with free Cu^{II} leads to subsequent copper incorporation, zinc release, and disulfide bond formation.[76] The same group later found that Zn_7MT-3 could interact with Cu^{II} bound to soluble and aggregated form of amyloid-β peptide.[77] In the presence of ascorbate, Zn_7MT-3 could prevent copper generated hydroxyl radical formation by quenching the redox activity of free or amyloid-β bound Cu^{II} ions.[76,77] This, along with the down regulation of MT-3 in these neurological disorders, has served to infer a role for MT-3 in protecting the central nervous system against copper-mediated toxicity. It should be noted that the final characterization of the copper content, oxidation state and cluster arrangements of the active form of MT-3 in vivo has yet to be proven.

Evidence for the Existence of Physiological Thionein

Historically, biological roles for T have been dismissed because, in vitro, it seemed to be degraded by proteases much more rapidly than the metal bound form. The zinc-bound thiolate groups of MT are chemically reactive when compared with those of other zinc proteins but they are considerably less reactive than those in metal-free T. As discussed, such MT/T redox systems implicate important physiological roles for the metal-free T in tissues.

Evidence for the existence of T was the result of studies of neoplastic cell lines.[78] Both the metal and thiol contents of the native protein have been measured along with the total protein. Comparison of the total metal and thiol concentrations of MT demonstrate the presence of T in the native sample. The observation that all seven zinc atoms in MT do not bind with the same strength suggests that MT may exist as a dynamic protein with different species for example, Zn_4MT, Zn_6MT and Zn_7MT, present.[24] Presumably the cysteine ligands not involved in metal binding in these species may be present as reduced thiols (T-like species, T_r) or disulfides (T_o).

Comparison of the total metal and thiol concentrations of MT show that the concentration of thiols exceed those of metal ligands, indicating the presence of metal-deficient MT species in the native sample.[78] Recently the development of assays for the detection of these metal-deficient species of MT, in both the reduced (T_r) and oxidized (T_o) form, have shown that, under physiological conditions, all three species are present in cells and that a significant proportion of MT exists as the metal-free species. Rat liver contains 27% total T (T_r+T_o), 26% of which is in the oxidized form, T_o. Rat kidney and brain contain greater than 50% metal-deficient MT (T_r+T_o).[79,80] The same investigators measured the zinc buffering capacity of human cancer cells, and determined that excess zinc induced T expression ensuring a surplus of zinc binding ligands.[81] Therefore, it appears that in cells, MT may exist as a dynamic protein with different species (for example, MT and metal-deficient T_r, and T_o) dependent on the state of the cell. The identification of metal-free T and its oxidized form, T_o, in cells shows that MT may undergo dynamic changes in vivo, and therefore act as a zinc buffer. This evidence implies that the MT/T redox system plays an essential role in controlling zinc delivery.

Conclusions and Outlook

The biological role of zinc was slow to be appreciated due to the lack of color of its salts and apparent absence of redox activity. However, the compatibility of zinc with sulfur and the formation of zinc/cysteine cluster motifs in MT has since been recognized to confer oxidoreductive properties on the bound zinc.[82] This is consistent with the frequent occurrence of cysteines as ligands in proteins as a means both for tight and weak binding of zinc through thiols and disulfides, respectively,[83] as well as the use of cysteine ligands as redox sensitive regulatory switches.[84] The control of cellular zinc distribution by a MT/T redox system can be related to the function of the energy state of the cell as witnessed by the interactions of MT/T with ATP, GSH and ROS and the reduction of MsrB3 by T. This represents one of the long sought roles of the MT/T system. In this regard, it would appear that MT might be analogous to ATP, which confers oxidative properties on inorganic phosphate.[85] MT might function through a similar redox mechanism, by distributing zinc to enzymes in metabolic networks including respiration and gene expression. These specifics, and those yet to be determined, refute the widely held belief that MT primarily scavenges radicals or detoxifies metals. The catalytic interactions of selenium and tellurium derivatives with MT may feature prominently in the development of Se/Te chemical or protein based therapeutic approaches to address cellular zinc imbalance.

It also seems possible that MT plays a role in neural activity, both by storing and distributing zinc for the neuronal network and protecting it against cellular damage. There is evidence that MTs expressed in astrocytes following CNS injury exhibit both neuroprotective and neurodegenerative properties and may be essential for recovery. Since these proteins lack signal peptides and have well-characterized free radical scavenging and heavy metal binding properties, the neuroprotective functions of MTs have been attributed to their intracellular roles.[86] However, recent work has suggested that extracellularly applied MT and a peptide modeled after MT might induce an effect on neurons in the brain through binding to neuronal receptors. If so, this would initiate signal transduction pathways resulting in neurite outgrowth and survival.[87]

The neural functions of MT are complicated by the observation that MT-3, which predominates in the central nervous system, is isolated with copper and zinc bound to separate domains. While the precise metal composition of MT-3 in vivo remains to be identified, the protein's rapid-dynamic structure might be central to its various roles in neurodegenerative diseases, in which both zinc and copper have been implicated. However, the characterization of physiological copper reactivity and regulation with regard to MT-3 remains inadequately defined.

The metal-sulfide bonds of MT clusters underlie all of these functions as judged by the unexpected consequences of the release of the redox inactive zinc from MT by physiological oxi-

dizing agents, the concomitant oxidation of sulfhydryl groups, and the transfer of zinc from MT to zinc-dependent enzymes.

Present evidence strongly suggests that the various forms of MT are unique to the specific needs of biology, particularly in maintaining oxidoreductive homeostasis. The remarkable composition and structure of this molecule guarantee the preservation and integrity of its function (that is, survival of life processes)—the major objective of the medical sciences. Ongoing spectroscopic studies fully support the above considerations and their extensions to the role of MT-3 in biological processes.[88]

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