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Review

Fluorescent probes for the structure and function of metallothionein☆

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ABSTRACT

Fluorescence methods have been instrumental in demonstrating that the structure of human metallothionein *in vivo* depends on the availability of metal ions and the redox environment. Differential chemical modifications of its cysteine thiols with fluorescent probes allowed determination of three states: metallothionein (zinc-bound thiolate), thionein (free thiols), and thionin (disulfides). Interrogation of its zinc-binding properties with fluorescent chelating agents revealed that the affinities for the seven zinc ions vary over four orders of magnitude. Attachment of fluorescent labels generated metallothionein FRET (fluorescence resonance energy transfer) sensors for investigating its structure and function in living cells.

Contents

1. Introduction

Among the amino acid side chains in proteins, the sulfhydryl group of cysteine has the most remarkable functional potentials. It can be a nucleophile, form disulfide bonds, or participate in other redox reactions, and importantly, it can serve as a ligand for metal ions. All of these potential functions are realized in zinc–thiolate interactions that are widespread in zinc metalloproteins, such as zinc finger proteins and zinc enzymes [1]. When interacting with zinc, the thiolate ligand retains some of its reactivity. This ligand-centered chemistry includes reactions with electrophiles/oxidizing agents as a way of mobilizing zinc ions from thermodynamically tight binding sites in proteins [2]. Owing to this reactivity, sulfur coordination environments can exist in at least three states, zinc-bound, zinc-free and reduced, and zinc-free and oxidized. This article describes approaches that were developed to distinguish these states in metallothionein (MT). The application of these approaches demonstrates that MT has a dynamic structure with regard to its metal content and redox state. This finding has important implications for the function of MT and the functions of zinc–thiolate interactions in other zinc metalloproteins.

2. Metallothioneins

Metallothionein was originally isolated from horse kidney and was identified as a protein with high sulfur and metal content [3,4]. It contained mostly cadmium and zinc and had only traces of other metal ions, such as copper. Fifty years after its discovery, we now acknowledge that there is a large family of MTs in eukarya and in some prokarya. Originally, MTs were assigned to three classes [5]:

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(i) proteins with sequences related to mammalian MT, (ii) proteins with sequences not related to mammalian MT, and (iii) peptides that are not genetically encoded. A subsequent classification did not include the third class. It focused on constructing an unrooted evolutionary tree of class I MTs, and it noted that class II MTs lack obvious homology to the mammalian forms and among each other [6]. These classifications are based on sequences and do not consider either the state of the thiols or the cargo, namely which metal is associated with the MT protein [7]. Moreover, the structural variability and diversity of class II MTs is so extensive that a clear basis for their classification has yet to be forthcoming, especially with regard to the fact that there is a scarcity of functional studies that would relate them to class I MTs.

Our work focuses on human MTs [8]. At least a dozen isoforms of MT exist in humans. In addition, there are polymorphic forms that have been associated with the risk of type 2 diabetes and its cardiovascular complications [9]. MTs are tissue- and isoform-specifically expressed by a variety of inducers. The scientific literature is replete with studies on changed expression of these proteins under a myriad of conditions. These studies address levels of either transcripts or total expressed protein, because most available methods do not provide information about the redox state of the thiols and the metal content of the protein. Hence, without further information about the state of the MT protein, conclusions about its functions remain speculative. Functions have been based on only one structural model of the protein. In this static model, 20 cysteines bind 7 zinc(II) ions in two zinc/thiolate clusters with bridging thiolate ligands (Fig. 1) [10,11]. At the center of the mechanism of action of MT, however, is the *dynamic* interaction of its cysteines with zinc and the redox activity of the sulfur ligand of the cysteines [12,13]. Discussions of MT functions need to examine this linkage between metal binding and the reactivity of the thiol ligands with oxidizing agents, such as disulfides and selenium compounds, carbonyls, and nitric monoxide, to name a few [14–18]. Antioxidant functions of MTs, including scavenging of reactive species, are associated with changed zinc ion concentrations that are potent effectors of biological processes. Likewise, metal binding affects the thiol reactivity and the redox-buffering capacity of MT with similarly significant consequences for cellular functions. These considerations are important for the pathophysiological and toxicological conditions of cellular exposure to additional metal ions or reactive species because, under these conditions, the physiological functions of the protein are compromised. The high degree of inducibility of MT makes it a potential biomarker for a significant number of diseases. Interpretation of the observed changes in expression of MTs hinges on the state of the protein.

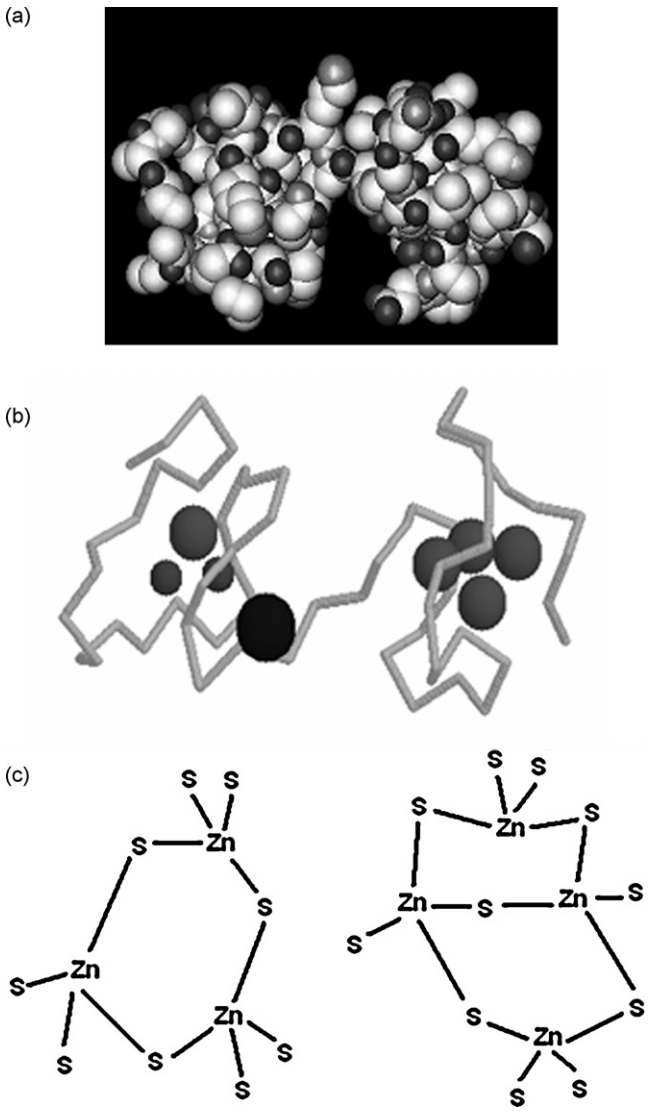

Fig. 1. The structure of mammalian metallothionein (isoform 2) as determined by X-ray crystallography and NMR spectroscopy [10,11]. (A) The protein envelops the metal ions completely in two domains. (B) Location of the metal ions in Cd_5Zn_2-MT crystallized from preparations of the protein from the liver of rats that had been treated with cadmium (protein database code: 4mt2). Four cadmium ions bind in the C-terminal α-domain; one cadmium ion (larger sphere) and two zinc ions (smaller spheres) bind in the N-terminal β-domain. A sodium ion is bound between the domains. (C) In Zn_7-MT, 7 zinc ions bind to the sulfur donors of 20 cysteines in the two zinc/thiolate clusters.

3. Characterization of the zinc-binding properties of MT with fluorescent chelating agents for zinc

The advent of chelating agents that are coupled to a fluorophore and become highly fluorescent when binding zinc(II) ions has provided decisive new tools for research in zinc biology [19–21]. We employed the agent FluoZin-3 to study the zinc-binding properties of human metallothionein-2. One zinc ion binds with only micromolar affinity ($\log K = 7.7$), two zinc ions bind with nanomolar affinity ($\log K \approx 10$), and four zinc ions bind with picomolar affinity ($\log K = 11.8$) [22]. This variation over four orders of magnitude is remarkable because, formally, all the zinc ions are in tetrathiolate coordination environments in the two clusters. However, the environments differ in the number of bridging thiolates. The three zinc ions in the N-terminal Zn_3S_9 cluster and two of the zinc ions in the C-terminal Zn_4S_{11} cluster have two terminal sulfurs and two bridging sulfurs. The remaining two zinc ions in the Zn_4S_{11} cluster have three bridging sulfurs. Thus, one consequence of the cluster structure is the lowering of the affinity for some of the zinc ions. As a result of these properties, metallothionein exists as different species: Zn_7T ("MT"), Zn_6T, Zn_5T, and Zn_4T. The distribution of these species depends on the total concentration of both the protein and zinc ions. Given the low affinity for the seventh zinc ion and the fact that cellular free zinc ion concentrations are only picomolar, Zn_7T cannot exist under normal physiological conditions [23]. This conclusion is supported by the observation that the protein takes up additional zinc when zinc ions are added to a liver homogenate [24]. Zn_6T and Zn_5T are the species that participate actively in zinc transfer from or to other proteins [24].

4. Analytical methods for identifying the state of the thiols in MT

The zinc-binding characteristics of MT suggest a dynamic role in controlling cellular zinc ion availability. Therefore, the state of

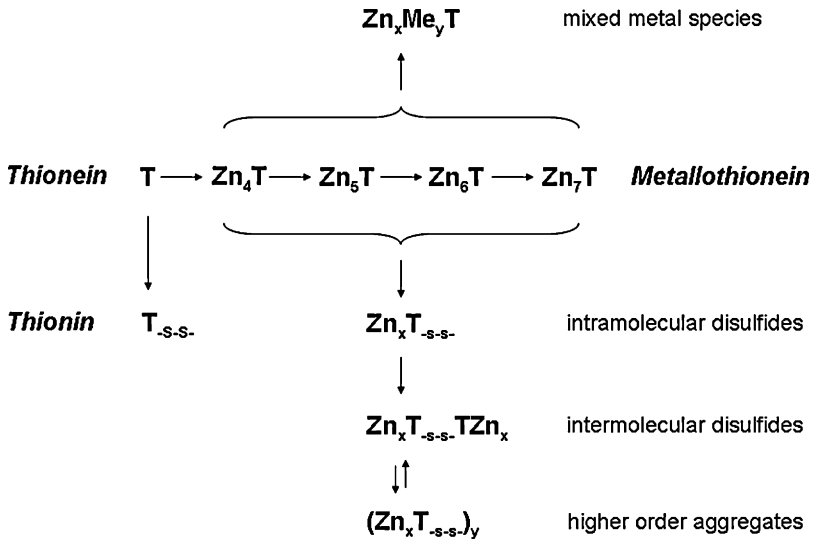

Fig. 2. Simplified differentiation of mammalian metallothionein forms in cells and tissues. The proteins exist in many different forms that differ in their metal load, redox state of the thiols, and degree of aggregation. Not shown are various modifications of the sulfur ligands, such as nitrosylation or glutathionylation, and non-covalent dimers.

the protein in the cell is a major issue. A comparison between the amount of Zn-MT and the amount of Cd-MT after cadmium was added to cell extracts suggests that Zn-MT is not saturated with zinc in some neoplastic cell lines [25]. Hence, we became interested in developing a method to determine the metal load of the protein by measuring both the apoprotein, thionein, and the holoprotein, metallothionein. We determined that thiol-reactive fluorogenic agents, such as 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) and its sulfonate, SBD-F, are especially well suited for chemical modification of thiols [26,27]. Based on increased reactivity of the thiols once a chelating agent removes zinc from MT, we devised a strategy for differentially labeling the thiols with ABD-F: labeling in the absence of EDTA would give the free thiols (thionein), while labeling in the presence of EDTA would give the total thiols (thionein plus metallothionein) [28]. By subtraction, the amount of metallothionein could be calculated. Critical to the analysis was the application of a non-thiol-based reducing agent, namely tris-(2-carboxyethyl)phosphine (TCEP), which reacts with disulfides but does not react with any other amino acid side chain and has very low affinity for zinc [29]. The complete procedure for analysis uses rapid modification of thiols in a cell or tissue extract, and then labeled MT is separated from other labeled proteins by HPLC on a reversed-phase Hypersil C4 column and detected fluorimetrically. Assays on liver, kidney, and brain tissue revealed thionein concentrations that are commensurate with those of metallothionein, thus demonstrating that the protein is not saturated with metal ions in the cell.

It is noteworthy that the analyses with ABD-F are performed under reducing conditions. Thus, any MT protein with oxidized sulfur ligands, if originally present, will escape detection. The redox activity of MT would seem to suggest that such forms of the protein, which we called thionin,[1] are present *in vivo* [15]. We, therefore, searched for conditions to identify such forms. We initiated studies with the three chemically defined species, metallothionein (loaded with 7 zinc ions), thionein (all 7 zinc ions removed and 20 reduced thiols), and thionin (all 7 zinc ions removed and all 20 thiols oxidized to disulfides) [30]. We used 6-iodoacetamidofluorescein (6-IAF) for rapid modification of thiols in the absence of TCEP. However, fluorescence of this agent is quenched almost entirely in the modified protein. Thus, absorption (ε_{492} = 81,000 M^{-1} cm^{-1}) rather than fluorescence was employed for detection. Employing chemical modification with 6-IAF in the absence and presence of TCEP, we could determine all three species in mixtures, but the method turned out not to be readily applicable for analysis of the state of MT in cells and tissues. For detection in tissues, we blocked all thiols (in the presence of EDTA) with *N*-ethylmaleimide, removed the excess reagent by extraction with ether, and then modified any remaining disulfides with ABD-F in the presence of TCEP. Using this procedure, we demonstrated that about 7% of the total thiols are oxidized in the protein and that this amount increases under conditions of oxidative stress [31]. Meanwhile, disulfides in MT were detected by isolating the over-expressed protein from the liver of transgenic mice, and from the same mice stressed by treatment with the drug doxorubicin, and subjecting the isolated protein to mass spectrometric analyses [32].

While these methods show the presence of at least three states, uncertainties remain about the structure of the protein under specific conditions, such as when the protein binds other metal ions or when other agents covalently modify its thiols. Sulfur oxidation states that are higher than disulfides and that cannot be reduced with TCEP would escape detection. Special analytical methods will be required to address these issues.

5. Analytical methods for electrophoresis and detection of MT

For analysis by SDS-PAGE, MT is generally carboxymethylated [33]. This procedure erases any information about its metal load and the state of its thiols. We developed a procedure for modifying the thiols with eosin-5-iodoacetamide (E-5-I) [34]. The advantage of this reagent is its high extinction coefficient (ε_{519} = 100,000 M^{-1} cm^{-1}) and the visualization of the protein during electrophoresis, thus obviating any staining after electrophoresis. We found that the protein polymerizes by successive addition of protomers [34]. Decamers were the highest aggregates that were detected with this technique. The extent of polymerization differs in the isoforms of rabbit MT-2 [35]. Thus, disulfides in MT can form both intramolecularly and intermolecularly. The significance of such supramolecular assemblies, which add yet another dimension to the number of states of the protein, is not known.

[1] Not to be confounded with thionin as a designation for disulfide-rich plant proteins of the defensin family or for a metachromatic dye (3,7-diamino-5-phenothiazinium acetate, Lauth's violet).

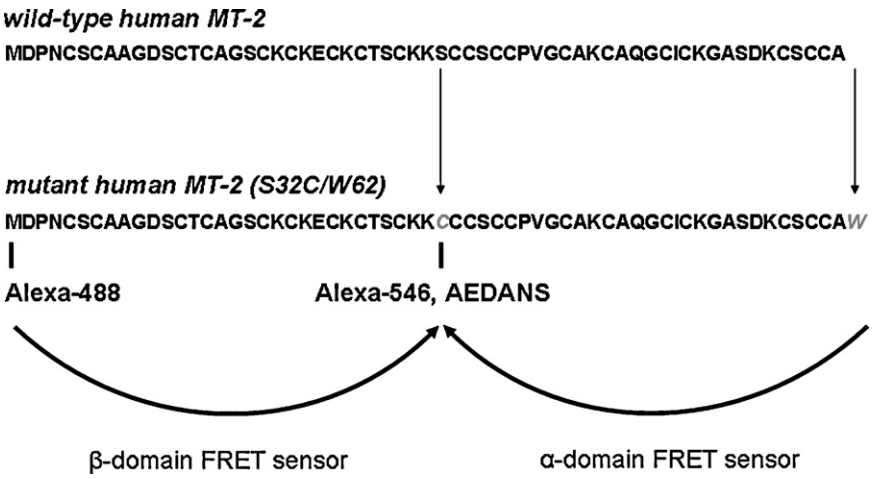

Fig. 3. Strategies for labeling human metallothionein with fluorescent probes. Two mutations were introduced into human MT-2: one cysteine between the two domains (S32C), a mutation that is found in human MT-1B, and an added tryptophan (W) at the C-terminus. Attachment of probes at the free N-terminal amino group and at the free thiol group at Cys-32 gives two FRET sensors for the metal load of the protein, one specific for the N-terminal β-domain, and another one specific for the C-terminal α-domain.

In conclusion, fully zinc-occupied metallothionein, fully reduced and metal-free thionein, and fully oxidized and metal-free thionin can be prepared *in vitro*. However, *in vivo*, "MT" is not fully loaded with zinc and the 20 cysteines are not fully reduced. In addition, it exists in polymerized forms. The name metallothionein, which traditionally stands for the structure of the protein with 20 reduced thiols and 7 bound metal ions, does not describe the state of the protein in cells and tissues (Fig. 2).

6. MT-FRET (fluorescence resonance energy transfer) sensors

Zn-MT is virtually devoid of spectroscopic characteristics. Mammalian MT has no aromatic amino acids and absorbs light only in the far ultraviolet region due to the charge transfer transitions of the Zn–S bond [36]. We, therefore, set out to attach fluorescent labels to MT. Compared to recombinant fluorescent proteins, an advantage of labeling proteins with small organic fluorophores is the brightness and photostability of these fluorophores. Also, fluorescent dyes modify the overall protein structure of a small protein, such as MT, much less than relatively large fluorescent proteins [37]. However, labeled proteins are not genetically encoded, and therefore, must be delivered to the cell by methods that involve protein transduction [38]. A very high-yield expression system based on the IMPACT expression system of protein splicing produces MT with a free N-terminus [39]. Labeling at the N-terminus can be preferential, because the N-terminal amino group has a lower pK_a value than the ε-amino group of the lysine side chain. Hence, choosing a pH value of 7.4 and using Alexa 488 succinimidylester allowed labeling of MT-2 with a 1:1 stoichiometry. For the construction of a FRET sensor, the protein needs a label at a second position. The fact that MT-1B has an extra cysteine in the linker region between the two protein domains suggested another position for labeling without interfering with the structure of the zinc/thiolate clusters. Hence, we used site-directed mutagenesis to engineer a cysteine into the corresponding position of human MT-2, resulting in the S32C mutant. Labeling is performed on the cadmium-containing protein, because the thiols in the clusters of zinc-containing MT turned out to be too reactive towards thiol-reactive probes. The cysteine in the linker region was labeled with Alexa 546 maleimide, also with a 1:1 stoichiometry. The double-labeled Cd-MT turned out to be a FRET sensor that is specific for metal binding in the N-terminal domain [40]. For the construction of a FRET sensor specific for the C-terminal domain, we employed a different strategy. Again, there was no suitable attachment point for a probe in this domain. We added a tryptophan as a fluorescence donor at the C-terminus of MT-2 (63W-MT), generating a mutant MT with light absorption in the ultraviolet region (280 nm). A suitable acceptor turned out to be {[(amino)ethyl]amino}naphthalene-1-sulfonic acid (AEDANS) attached to the cysteine in the linker region in the S32C/63W double mutant [41]. In the two sensors, the labels are attached in such a way that they do not interfere with zinc binding to the clusters (Fig. 3). After the cadmium-containing protein is labeled, cadmium needs to be removed and zinc introduced in order to prepare the zinc protein. For application of the Förster theory for distance determination from energy transfer measurements, the labels must rotate freely. Fluorescence polarization anisotropy measurements showed that the AEDANS label in S32C/63W MT-2 interacts with the protein [41]. Therefore, we presently pursue the production of a new generation of sensors by employing additional strategies for attachment of fluorophores and by using fluorophores with more favorable physical properties for energy transfer measurements.

7. Analytical biochemistry of human MTs

The two general approaches to investigating mammalian MT at the protein level are its isolation from tissues (mostly liver), or from bacteria that express the recombinant protein, and its direct characterization in tissues and cells. Since isolated MT is generally heterogeneous, it is converted to a form with 20 reduced cysteines and 7 bound divalent metal ions [42].

The majority of investigations on human MT isoforms in tissues and cells have been performed on the mRNA level. Immunological methods and metal saturation assays are routinely used to determine the concentrations of MT proteins. Cross-reactivity of commercial antibodies against the human MT isoforms is virtually unexplored. A cadmium saturation assay is based on the strong affinity of MT for this metal, the relative robustness of the protein against low pH values and high temperatures, and the scavenging of excess cadmium by hemoglobin [43]. These assays do not provide information about the relative amounts and characteristics of the isoproteins.

In human liver, two major isoforms, MT-1 and MT-2, are separable based on charge by either chromatography or electrophoresis [44]. Chromatography involves gel filtration on Sephadex G-50 followed by ion chromatography on DEAE-cellulose. At least five isoforms of MT-1 – six when including MT-2 – can be separated from

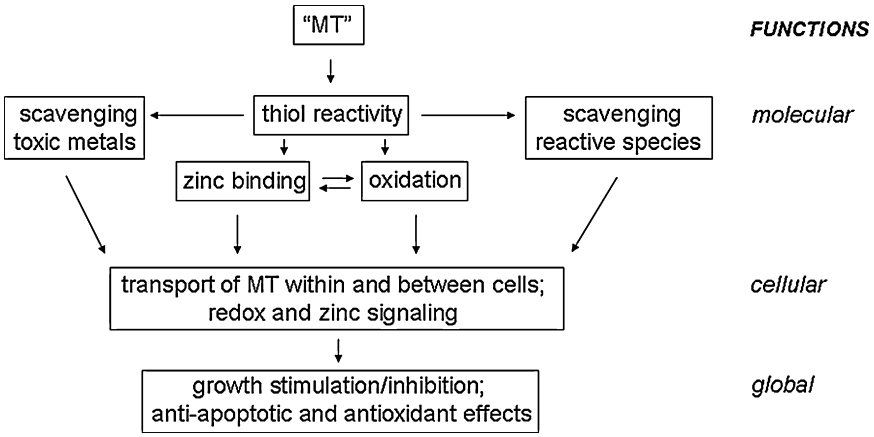

Fig. 4. The functions of mammalian metallothionein ("MT") presented in the form of a directed acyclic graph. Functions are defined at different levels. The focus of inquiries is on how chemical and biological activities are linked.

human liver by chromatography on an Aquapore RP-300 (Applied Biosystems) column or on reversed phase C-4, -8, or -18 columns [45]. Without sequencing the peptides, assignment to specific gene products is not achievable routinely. Hyphenated techniques, such as microbore reversed phase (RP) HPLC–ICP MS and capillary zone electrophoresis (CZE)–ICP MS for element-specific detection combined with the same chromatographic and electrophoretic techniques coupled with ES MS for molecule-specific detection provide great promise for quantitation, identification, and characterization of human MT isoforms [46,47]. These techniques, in one-dimensional or multidimensional versions, combine the selectivity of separation techniques and the sensitivity and structural information obtained by mass spectrometry [48]. They have been employed to characterize MTs from various species, but rarely have they been employed to investigate human MTs, where at least a dozen isoforms and a yet unknown number of polymorphisms impeded progress. In human brain cytosol, MT-1, MT-2, and MT-3 can be separated by CZE–ICP MS and analyzed for their zinc, cadmium, and copper content [49]. Using anion exchange and size exclusion chromatography coupled with atomic emission spectrometry, the same metals were also determined in MT from human liver cytosol [50].

8. Conclusions

The isolation and characterization of MT has met with unparalleled challenges and provides an important lesson on how interventions by the investigator influence the outcome of analytical procedures. Since the isolated protein is generally heterogeneous, investigators prepared a homogeneous form *in vitro* by fully reducing the cysteines, removing the bound metals, and re-constituting the protein with seven divalent metal ions. The recognition that MT is sensitive to oxidation resulted in the addition of thiol-reducing agents, such as 2-mercaptoethanol, during its isolation. These procedures neglect the possibility that the heterogeneity in its redox state and metal content is an inherent property of the protein. While re-constituted forms provided valuable material for biophysical characterization, they precluded further insights into the state of the protein *in vivo*. What has been poorly appreciated in the purification of MT is the cellular and subcellular location of the protein. MTs are not only cytosolic but are transported to the nucleus and to the intermembrane space of mitochondria [51,52]. They are also not exclusively intracellular. MTs are exported from cells and taken up by cells through a receptor-mediated mechanism, in which the protein remains in an endocytotic compartment and the metal is translocated to the cytosol [38,53,54].

For the analytical characterization of the MT protein, controlling the redox state and the concentration of both metal ions and the protein is similarly critical. The presence of chelating or redox-active agents, or simple dilution of the protein, can change its state. The separation of the dozen human isoforms in combination with detection methods for the state of their thiols, metal composition, and metal load continues to be challenging.

The sensitivity and selectivity of fluorimetric methods allowed analysis of the state of the MT protein. In the past, interpretations were based on two premises that now can be dismissed, namely that cellular zinc ions are freely available at high enough concentrations to saturate the binding sites of thionein, and that oxidative chemistry of zinc/thiolate coordination sites cannot occur in the largely reducing environment of the cytosol. Studies of both the isolated protein and the protein in tissues and cells arrive at the same conclusion, namely that MT exists in different oxidation states and with different metal loads. Determination of these states of the protein is a basis for discussing its chemical activities and biological functions in zinc and redox metabolism (Fig. 4) [55,56]. Depending on their states, the functions of MT proteins can be opposite and result in conflicting observations from different experiments. A highly metal-loaded protein can donate zinc, while a protein with a low metal load would rather accept zinc. Likewise, the redox state of its thiols will determine whether the protein is a reductant or an oxidant. Similar caution should be exercised when interpreting the role of the protein in pathophysiology, because its state changes when it is over-expressed or down-regulated, and it may well have the opposite effect to what is inferred from the one three-dimensional structure of MT. The high reactivity of the protein with some metal ions and with thiol-reactive substances offers new perspectives on the conditions under which the analytical characterization of metallothionein should be performed and how data are to be interpreted. The fluorimetric methods discussed here are exemplary for exploration of other proteins with zinc/thiolate coordination environments that participate in redox reactions and function in transient zinc binding.

Acknowledgments

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