Metallothionein Redox Cycle and Function

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The biologic function of metallothionein (MT) has been a perplexing topic ever since the discovery of this protein. Many studies have suggested that MT plays a role in the homeostasis of essential metals such as zinc and copper, detoxification of toxic metals such as cadmium, and protection against oxidative stress. However, mechanistic insights into the actions of MT have not been adequately achieved. MT contains high levels of sulfur. The mutual affinity of sulfur and transition metals makes the binding of these metals to MT thermodynamically stable. Under physiologic conditions, zinc-MT is the predominant form of the metal-binding protein. The recognition of the redox regulation of zinc release from or binding to MT provides an alternate perspective on biologic function of MT. Oxidation of the thiolate cluster by a number of mild cellular oxidants causes zinc release and formation of MT-disulfide (or thionin if all metals are released from MT, but this is unlikely to occur *in vivo*), which have been demonstrated *in vivo*. Therefore, the thermodynamic stability of zinc binding makes MT an ideal zinc reservoir *in vivo*, and the redox regulation of zinc mobilization enables MT function in zinc homeostasis. MT-disulfide can be reduced by glutathione in the presence of selenium catalyst, restoring the capacity of the protein to bind zinc. This MT redox cycle may play a crucial role in MT biologic function. It may link to the homeostasis of essential metals, detoxification of toxic metals and protection against oxidative stress. Exp Biol Med 231:1459–1467, 2006

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Introduction

Metallothionein (MT) is a transition metal binding protein that is found in all eukaryotes and in some prokaryotes (1–3). Ever since its discovery in 1957 (4), MT has been constantly studied in all aspects, including its physical, chemical, and biochemical properties and biologic function (5–7). The most important characteristic of MT that confers the unique biologic significance of this low–molecular weight protein is that it is cysteine rich. Among the amino acid compositions of mammalian MT, 20 are cysteines (5–7). This unique protein is implicated in a diversity of intracellular functions (8), but the only consensus among MT researchers thus far concerns its role in the detoxification of heavy metals, which is due mostly to its high affinity with these metals (9, 10). In mammals, MT predominantly binds zinc (11), but under the condition of copper or cadmium overload, zinc can be readily displaced (12). Cells that contain an excess amount of MT are resistant to cadmium toxicity (13), whereas cell lines that cannot synthesize any MT are sensitive to cadmium (14). Genetic studies using transgenic or knockout mouse models further demonstrate the role of MT in protection from cadmium toxicity (15–17). However, it has been argued that MT likely performs an evolutionarily conserved function rather than simply protecting from toxicity induced by man-made metal pollution (18, 19).

The binding of zinc to MT has proven to be physiologically relevant. Several studies have produced strong evidence to support the idea that MT functions as a zinc chaperone for the regulation of gene expression and activity of proteins such as metalloproteins and metal-dependent transcription factors (20–24). The binding of zinc to MT is thermodynamically stable, which makes MT an ideal zinc reservoir *in vivo*. The question is how MT makes zinc available for other molecules, including transcription factors and metalloproteins.

The most critical advance in MT research is the demonstration of the redox regulation of Zn-S interaction and the coupling of zinc and redox metabolism (25, 26). The cluster structure of Zn-MT provides a chemical basis by which the cysteine ligand can induce oxidoreductive properties (27). This structure allows for thermodynamic stability of zinc in MT while permitting zinc to retain kinetic lability. The sulfur ligand confers the redox activity on the zinc-MT complex and can be oxidized and reduced with concomitant release and binding of zinc in an oxidoreductive environment. The release of zinc from MT makes zinc available for the functional demand of other molecules. The reduction of the oxidized MT restores the zinc binding capacity and, thus, the repletion of the endogenous zinc

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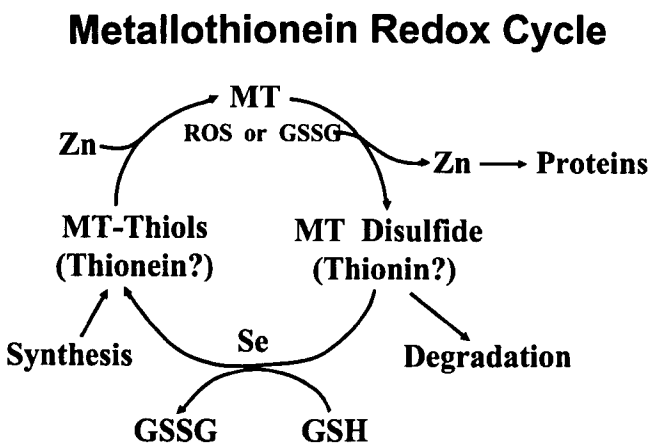

Figure 1. Schematic presentation of MT redox cycle. Under physiologic conditions, zinc bound to MT is released through oxidation of the thiolate cluster when the environment becomes oxidized. Formation of MT-disulfide or thionin (if all metals are released from MT) follows zinc release. MT-disulfide or thionin would be subjected to degradation, however, when the oxidized environment became reduced—through, for example, an increase in GSH/GSSG ratio—MT-disulfide or thionin is reduced to MT-thiol or thionein. This reduction process is greatly enhanced in the presence of selenium catalyst or other unidentified catalytic agents. In the presence of zinc, MT is quickly reconstituted. This process constitutes MT redox cycle, which plays a crucial role in the biologic function of MT.

reservoir. This process constitutes a MT redox cycle, as shown in Figure 1, which provides an alternate perspective on biologic function of MT.

Zinc Release from MT

Early studies have shown that there is fast zinc exchange between MT isoforms (24), between MT and the zinc cluster in the Gal4 transcription factor (24), and between MT and the apoforms of various zinc proteins (23, 28). Importantly, zinc release from MT is modulated by both glutathione (GSH) and glutathione disulfide (GSSG) (20, 28, 29). GSH inhibits zinc release in the absence of GSSG, indicating that MT is stabilized at relatively high cellular GSH concentrations. The presence of GSSG results in zinc release. The rate of zinc release depends linearly on the amount of GSSG: the more oxidative the redox state becomes, the more efficiently zinc is released from MT (27). Moreover, cellular disulfides other than GSSG also react with MT to release zinc with high efficiency (20).

Mobilization of zinc from MT by an oxidative reaction may either constitute a general pathway by which zinc is distributed in the cell or be restricted to conditions of oxidative stress in which zinc is needed in antioxidant defense systems (20). Under stress conditions, zinc release from MT occurs when the levels of nitric oxide or reactive oxygen species increase (30–33). For example, treatment of lung fibroblasts with *S*-nitrosocysteine (SNOC) results in an increase in intracellular labile zinc, as detected by a zinc-specific fluorophore Zinquin, in wild-type (WT) but not MT-null fibroblasts (31). Furthermore, in an elegant study Pearce *et al.* have shown that in cultured pulmonary artery endothelial cells an MT-green fluorescent fusion protein (FRET-MT) undergoes conformational changes in the presence of NO (34). These conformational changes are consistent with the release of metals from the thiolate clusters of MT (25–27).

Zinc movement in the cell is tightly regulated, and free zinc in the cell is very limited (35). Intracellular zinc is an integral component of cytoskeletal structures, transcription factors, and metalloenzymes (19). However, normal cellular activity requires zinc mobilization and transfer from one location to another or from one zinc-binding site to another. In cell-free systems, studies have shown that zinc transfers from MT to carbonic anhydrase (36), sorbitol dehydrogenase (28), alkaline phosphatase, and bovine carboxypeptidase (23). Zinc exchange between MT and zinc finger transcription factors (37–39) serves as a mechanism for the regulation of gene expression through activation or inhibition of DNA binding by estrogen receptors (40), SP1 (22, 38), TFIIIA (21, 39), Gal4 (24), or tramtrack (41). In addition, a direct interaction between MT and apo-zinc binding peptides during the process of zinc transfer has been demonstrated in a cell-free system (42).

A recent study demonstrated that zinc transfer from MT to other proteins occurs *in vivo* (43). Heart extracts obtained from MT-null mice were incubated with ^{65}Zn-MT or $^{65}ZnCl_2$ to identify differentially labeled ^{65}Zn proteins. The proteins receiving ^{65}Zn were separated by blue-native polyacrylamide gel analysis (BN-PAGE), or sodium dodecyl sulfate–PAGE (SDS-PAGE). A unique ^{65}Zn-binding band was only observed from the ^{65}Zn-MT–incubated but not the $^{65}ZnCl_2$-incubated preparation. Further analysis using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer revealed that mitochondrial aconitase (m-aconitase) was among the proteins accepting Zn directly from Zn-MT. The m-aconitase, not the cytosolic aconitase (c-aconitase), was co-immunoprecipitated with MT. Thus, MT indeed transfers zinc to other proteins *in vivo*, and some of these transfer processes require a direct interaction between MT and the zinc acceptor proteins.

There are two major concerns regarding zinc transfer from MT to other proteins. The first is the energetic feasibility of zinc transfer from MT to other proteins, and the second is the specificity of zinc transfer. As discussed above, the thermodynamic barrier could be overcome *via* redox reaction at the zinc site of MT thiolate clusters. The conformational changes induced by the protein-protein interaction between MT and the zinc acceptor proteins would provide another means to achieve the lability of zinc bound to MT. A direct interaction between MT and the zinc acceptors has been demonstrated when an apo-zinc binding peptide and Zn-MT were separated by a membrane through which zinc ions could diffuse but the peptide or MT could not (42). There was no detectable zinc-bound peptide found after a 3-hr incubation. However, the transfer occurred in

less than 2 mins when the apopeptide and Zn-MT were mixed (42).

The direct interaction between MT and zinc acceptor proteins would ensure the specificity of zinc transfer under demanding conditions. This would represent a unique regulatory mechanism by MT of zinc homeostasis. Given that the binding of zinc to MT is thermodynamically stable, the bound zinc would then only be delivered to specific proteins through a direct interaction between MT and the zinc acceptors. This specific zinc delivery mechanism could ensure that the function of zinc-binding proteins is appropriately regulated in response to changes in the intracellular environment. In this context, MT would serve as a zinc-specific chaperone, delivering zinc to a specific group of proteins. It would appear that m-aconitase is among the zinc acceptors. Other proteins that may receive zinc specifically from MT are yet to be identified. In this context, metabolic disturbances in MT-null mice might be expected. However, many studies have not identified obvious detrimental phenotypes in MT-null mice in which MT-I and MT-II genes were interrupted (18), although one report has shown that MT-null mice develop obesity (44). Thus, it is possible that some proteins may require MT as zinc chaperone to receive zinc, but in the absence of MT other alternative chaperones or compensatory mechanisms may overcome the deficiency to prevent potential detrimental consequences due to the lack of MT.

Zn release from and binding to MT should result in a dynamic change in the level of Zn bound to MT. In conjunction with this change, MT becomes oxidized. These events recently have been observed in intact animal studies (45).

Formation of MT-Disulfides

Attempts to demonstrate the formation of MT-disulfide bonds *in vivo* were impractical in the past, largely due to the fact that the tissue concentrations of MT were insufficient. This limitation is now overcome by the availability of MT-overexpressing transgenic mice (46). A strategy of differential alkylation of cysteine residues in MT to identify disulfide bond formation *in vivo* has been developed (45). A pH of 6.0 and nitrogen-flushed buffer is used for tissue homogenization immediately after tissue harvest, in the presence of *N*-ethylmaleimide (NEM), to trap the sulfhydryl groups of cysteine residues that are not present as disulfide bonds *in vivo*. Then, after reduction of the disulfide bonds with dithiothreitol (DTT), radiolabeled iodoacetamide (IAA) is used to alkylate the cysteine thiols. If MT-disulfide exists, an incorporation of ^{14}C will be observed in the presence, but not in the absence, of DTT.

Using the method above, a recent study (45) has uncovered the presence of MT-disulfides under both physiologic and oxidative stress conditions in the form of intramolecular but not intermolecular disulfide. Both α- and β-domains of MT are involved in the disulfide bond formation. Zinc release from MT or the formation of disulfide bonds in MT is a random process, and the number of zinc atoms released or the number of disulfide bonds formed varies from time to time, but these increase when the environment becomes more oxidized.

It is not for MT-disulfide bonds to present under physiologic conditions. However, this provides an important insight into the biologic function of MT. Under either physiologic or oxidative stress conditions, redox regulation of metabolism and signal transduction is a vital aspect of cellular function. Since zinc release from MT is regulated by oxidoreductive conditions, biochemical changes in the MT molecule are expected to occur under both physiologic and stress conditions. Such a change involves MT-disulfide bond formation, and increased disulfide bond formation in MT is expected under oxidative stress conditions because additional zinc release occurs in response to oxidative stress.

Structurally, the 20 cysteines of MT are sequentially very close, which seemingly would favor intramolecular disulfide bond formation (30). A number of *in vitro* studies have shown that MT can form dimers through disulfide bond formation under oxidative conditions (30, 37, 47, 48), although intramolecular oxidation of the cysteine thiolates and mixed disulfide formation also have been demonstrated (37). The factors that control the partitioning between intermolecular and intramolecular disulfide bond formation under *in vitro* or *in vivo* conditions are largely unknown. One possibility is that the MT concentration *in vivo* is significantly lower than that *in vitro*, thus favoring intramolecular disulfide bond formation *in vivo*.

The thiol groups in MT are masked by their coordinated metal ions, but they retain a substantial degree of the nucleophilicity seen with the metal-free protein (30). Structural studies of sulfur reactivity have shown that disulfide bonds could be formed in both the α- and β-domains (30). However, *in vitro* studies have often shown that the β-domain is involved in disulfide bond formation under oxidative or nitrosative stress conditions. A recent *in vitro* study showed that exposure of MT to NO led to selective release of three metals from the β-domain while leaving the four metals in α-domain unchanged (49). In the study mentioned above, both the α- and β-domains of MT are involved in disulfide bond formation under both physiologic and oxidative stress conditions (45). It has been suggested that the lability of each cluster toward various reagents is likely to be controlled by the specific steric and electronic factors that vary from reagent to reagent (37).

It is important to note that zinc release from MT under either physiologic or stress conditions occurs in a random manner. However, the first five cysteines in the N-terminal α-domain likely are resistant to oxidation. A tandem MS/MS spectrometry study has shown that cysteine 44 and cysteine 48 often are involved in disulfide bond formation *in vivo* (45). This observation does not support the notion derived from *in vitro* studies that only the β-domain of MT is important for homeostasis of zinc and copper (50). It

appears that the number of zinc atoms released from one MT molecule at a time varies, although the possibility that all seven zinc atoms can be released at once cannot be excluded.

A study using a procedure for differential fluorescence labeling of cysteine clusters has revealed the presence of free thiols in MT under physiologic conditions (51). The study used a 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide to react with cysteine residues that are not bound to zinc. A fluorescent adduct is formed through the reaction, which can be detected quantitatively. But the zinc-bound cysteine residues or disulfides do not react. The analysis by this procedure revealed almost as many MT molecules that react with the fluorescence as molecules that do not. Thus, this study demonstrates that as much as 50% of the total cysteine residues in MT may not bind to zinc or other metals under physiologic conditions. However, the method used cannot distinguish thionein (an apoform of MT that has no metals) from MT-thiols (a mixture of the cysteine residues in MT in the form of either metal-binding or non–metal-binding thiols). Another limitation of the method is that it cannot detect disulfide bond formation in MT. The study using tandem MS/MS spectrometry detected the presence of disulfide bonds in MT (45). Together, these studies demonstrate that thiols in MT undergo a dynamic change, with a concomitant release or binding of zinc under redox regulation. This process involves both the formation and reduction of MT-disulfides.

Reduction of MT-Disulfides

Coordination of zinc to the thiol group of cysteine residues in MT and the low redox potential of the thiolate clusters make the coupling between zinc and redox metabolism possible. A number of mild cellular oxidants can oxidize the thiolate clusters, leading to zinc release (27–29). Among the physiologic oxidants is GSSG. It has been shown that changes in the GSH/GSSG ratio modulate zinc transfer from MT to other zinc-binding proteins (28). Therefore, the changes in the cellular redox state serve as a signal for zinc mobilization and distribution through the redox reaction of thiol clusters in MT.

After zinc release, the fate of MT-disulfides is a critical issue in MT metabolism and function. One possibility is to reduce MT-disulfides to MT-thiols or MT. Several studies have examined the reduction of MT-disulfides *in vitro* (47, 52–54). Addition of GSH to a system containing MT-disulfides induced by air can cause an observable reduction of MT-disulfides (52). Interestingly, chemical reducing agents such as 2-mercaptoethanol or dithiothreitol cannot reduce MT-disulfides induced by hydrogen peroxide (47). However, another study has shown that treatment with dihydrolipoic acid almost completely reduced the oxidized thiols in MT exposed to hydrogen peroxide (53). A fundamental question is whether or not MT-disulfides can be reduced *in vivo*.

An elegant study has specifically addressed the reduction of MT-disulfides by physiologically relevant reducing agents (54). In the assay system, MT was oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid), and the release of zinc from MT was measured spectrophotometrically by the formation of a zinc-PAR. After oxidation, GSH and selenocystamine were added to the system, and the reduction of MT-disulfides was monitored by the dissociation of zinc-PAR. It was observed that GSH alone removed only small amount of zinc from the zinc-PAR complex, but the combination of GSH and selenocystamine could remove 85% of the total zinc originally released from MT from the zinc-PAR complex in 1 hr. When MT was oxidized with hydrogen peroxide, the same physiologically relevant reducing agents, GSH and selenocystamine, efficiently reduced the disulfide. However, 2-mercaptoethanol cannot have a significant reducing effect, suggesting a selective action between physiologically relevant reductants and MT-disulfides. Moreover, if the ratio of GSH to GSSG decreases, the reduction reaction is inhibited, or zinc release from MT, instead of binding to MT, becomes dominant.

The use of the dissociation of zinc-PAR as an indicator of reduction of oxidized MT allows observation only of the restoration of zinc binding capacity, presumably due to the reduction of MT-disulfides. It cannot indicate whether or not MT-disulfides can be reduced to MT-thiols or thionein. However, this limitation does not diminish the significance of the reduction of MT-disulfides by physiologically relevant reducing agents. The change in GSH/GSSG ratio is a physiologic process of cellular redox reactions, which signals cellular responses such as gene expression and modification of protein functions. High GSH/GSSG ratios prevent oxidation of MT and promote zinc binding to the thiolate clusters, and low ratios stimulate zinc release and MT oxidation. Thus, this process constitutes the MT redox cycle and facilitates intracellular zinc metabolism and function. It will be important to demonstrate that the reduction of MT-disulfides actually occurs *in vivo*.

Possible Link Between MT Redox Cycle and Biologic Function

Many studies have implicated three major biologic functions of MT, including homeostasis of essential metals, detoxification of toxic metals, and protection from oxidative stress. However, the lack of a sufficient mechanistic understanding of the action of MT makes the study of biologic function of MT progress with uncertainty. The MT redox cycle described above may provide an alternative perspective on this perplexing topic. The chemical properties of the MT redox cycle have potential links to the biologic function.

MT Redox Cycle and Zinc Homeostasis. Several zinc transporters have been identified and characterized and they participate in mammalian zinc metabolism by transporting zinc across plasma membranes (55). These proteins

belong to two solute-linked carrier (SLC) gene families, *ZnT (SLC30)* and *Zip (SLC39)*. All of these proteins have transmembrane domains and it appears that ZnT proteins transport zinc from cytosol to extracellular space or to intracellular vesicles and Zip proteins promote extracellular zinc uptake and zinc release from vesicles to cytoplasm. As described above, zinc movement in the cell is tightly regulated and intracellular free zinc is very limited (35). However, these zinc transporters are all associated with plasma membranes. Therefore, there must be an intracellular zinc trafficking regulation, by which excess zinc can be transferred to ZnT proteins and zinc entry via Zip proteins can be delivered to the targets. MT and the MT redox cycle thus can effectively serve this cellular function.

The ubiquity of MT in mammalian cells facilitates its interaction with other molecules, perhaps, including zinc transporters. In addition, there are multiple isoforms of MT in mammalian systems (1–3, 19). Therefore, the ensured availability of MT via its ubiquity and duplication of isoforms makes it an ideal zinc chaperone in the cell. The thermodynamic stability of zinc binding to MT ensures the intracellular zinc reservoir function of MT and the redox mobilization of zinc through oxidation of the thiolate cluster makes zinc delivery to target proteins possible. Several proteins that receive zinc from MT have been identified (23, 28, 36–39), as discussed above. It will be important to demonstrate whether or not MT directly interacts with zinc transporters *in vivo*.

The synthesis of MT in cells is highly inducible by zinc load. This further facilitates the zinc reservoir function of MT. Excess zinc in the cell can be sequestered by elevation of MT levels and zinc-MT would facilitate zinc efflux by interacting with ZnT proteins. The regulation of zinc trafficking in the cell under the condition of zinc load or zinc deficiency would be fundamentally different. These regulation mechanisms would involve the interaction between MT and zinc transporters, both ZnT and Zip proteins, and possible gene regulation by MT of the zinc transporters.

It has also been found that MT can be transported into mitochondria (56) and nuclei (57–59). MT transport into these organelles is linked to zinc movement and the subsequent effect on mitochondrial respiratory function (56), gene regulation (57) and control of cell proliferation and differentiation (58, 59). The role of MT in the regulation of zinc homeostasis can be recognized at least in two fundamental processes of zinc metabolism and transport. First, MT facilitates the movement of zinc in the cell as well as delivers zinc to the target molecules on demand. Second, MT makes zinc available to crucial organelles such as mitochondria and nuclei, in which the identified zinc transporters have not been shown to function.

Most studies examining the role of MT in mineral homeostasis have focused on zinc mobilization and transfer. However, it has been well known that MT binds to copper and appears to participate in the regulation of copper homeostasis (60). It will be important to determine the specificity of MT binding to different metals under different conditions *in vivo* and the interrelation among the metal-bound MTs.

MT Redox Cycle and Metal Detoxification. The earliest suggested and most consented function of MT is its role in detoxification of metals. The discovery of MT was associated with cadmium binding (4). Since then, the effect of MT on cadmium metabolism and toxicity has been studied extensively. Such studies continue along with advances in biochemical and molecular techniques and instrumentation invention. The binding of cadmium to MT and the structural characteristics of cadmium-MT have been a topic since the discovery of MT (61, 62). These structural studies have unambiguously demonstrated the interaction between cadmium and MT.

Genetic experiments using MT-transgenic or MT-knockout mouse models have provided direct evidence that MT protects from cadmium toxicity (15–17). Theses studies confirmed previous *in vivo* observations using MT inducers and cultured cells (12–14). However, the mechanistic insights into MT detoxification of cadmium toxicity are still missing. It has been shown that cadmium can dissociate from MT through the same oxidoreductive reaction described above for zinc release. Cadmium transporters, if any, in mammalian systems have not been identified. As a cadmium sink, MT could eliminate cadmium interaction with other cellular components. However, intracellular release of cadmium from MT in response to redox changes would make cadmium re-appear if it were not effectively transported from the cell. Some early studies have shown that cadmium exposure induces MT synthesis in liver, and MT in turn binds to cadmium to reduce its distribution to critical macromolecules in liver (63–65). Interestingly, with long-term exposure to cadmium, kidney injury becomes a dominant form of target organ toxicity of cadmium. It has been shown that renal toxicity is induced by cadmium-MT that is released from liver and taken up by kidney (65). The efflux of the metal-MT complex is an interesting topic that requires more comprehensive understanding.

Besides regulating copper homeostasis, MT also may participate in copper detoxification. Copper detoxification by MT may be operated by different mechanisms relative to those of cadmium. Copper transporters have been identified in mammalian systems (66), and copper proteins that can function as copper chaperones also are available (67, 68). MT can function as a temporary sink of copper under overload conditions; then, through interaction with copper chaperones or copper transporters, it facilitates copper transport out of cells or into vesicles. The possibility that a direct transport of copper-MT across cell membranes also exists, and it will be important to demonstrate this *in vivo*.

An important note is that under cadmium and copper overload conditions, zinc bound to MT can be displaced readily. The higher affinity of MT to copper and cadmium than to zinc makes MT more thermodynamically favorable

to bind copper and cadmium. It has not been addressed whether or not this displacement of zinc binding to MT by either copper or cadmium can lead to disturbance in zinc metabolism. It is possible that detoxification by MT of copper or cadmium toxicity would pay the price by compromising zinc homeostasis. Many experimental studies, however, have examined the role of MT in metal's toxicity by acute exposure. The consequence of disturbance in zinc homeostasis would have a chronic effect, which should be an important consideration in the study of MT detoxification of heavy metals.

MT Redox Cycle and the Antioxidant Function of MT. The hypothesis that MT functions as an antioxidant against reactive oxygen and nitrogen species has received extensive experimental support from many *in vitro* studies. Studies using a cell-free system have demonstrated the ability of MT as a free radical scavenger (69–71). The first suggestion of the antioxidant function of MT was obtained from a study examining the effects of MT on the radiosensitivity of a cultured human epithelial (HE) cell line and mouse fibroblast (C1 1D) cells (72). Further studies showed that MT containing zinc and/or cadmium can scavenge hydroxyl and superoxide radicals produced by the xanthine/xanthine oxidase reaction system (69). All 20 cysteine sulfur atoms are involved in the radical quenching process, and the rate constant for the reaction of hydroxyl radical with MT ($K_{.OH/MT} = 2700$) is about 340-fold higher than that with GSH ($K_{.OH/GSH} = 8$) (69). Further studies have shown that MT is about 800 times more potent than GSH (on a molar basis) in preventing hydroxyl radical–generated DNA degradation *in vitro* (70).

Studies using cultured cells and intact animal models have provided further evidence supporting the antioxidant function of MT (73–80). These studies used different MT inducers to increase MT levels before application of oxidative stress induced by different reactive oxygen species generation systems. The most convincing evidence for the antioxidant action of MT was generated from genetically manipulated mouse model studies. Using MT-overexpressing transgenic or MT-knockout mice, many studies have shown MT protection against oxidative injuries induced by a diversity of oxidative conditions, including adriamycin treatment, alcohol administration, diabetes, and ischemia/reperfusion (81–90).

That MT directly reacts with all reactive oxygen species has been demonstrated only in studies using cell-free systems (69–71). It is questionable whether these *in vitro* observations are applicable to the *in vivo* action of MT. In particular, all of the reactive oxygen species, especially hydroxyl radicals, are very reactive and have an extremely short half-life. It is speculated that MT can only be effective as a free radical scavenger *in vivo* if it is located sufficiently close to the site of production of the radicals. This requirement of proximity thus predicts that the direct interaction between MT and the radicals as a major mechanism of action *in vivo* is impractical.

It has been argued that the primary determinant of MT protection against oxidative stress is zinc released from MT and its subsequent uptake by plasma membranes, since zinc protects against lipid peroxidation and thereby stabilizes membranes (91, 92). In addition, zinc may suppress lipid peroxidation by affecting many different cellular functions, such as decreasing iron uptake and inhibiting NADPH–cytochrome *c* reductase (93). A more compelling explanation of the antioxidant function of MT may come from the MT redox cycle. Importantly, the thiolate clusters of MT only react with physiologically relevant oxidants such as GSSG, highlighting the biologic significance of MT as an antioxidant. It is possible that MT functions as an antioxidant *in vivo* through its reaction with cellular oxidants, which in turn may directly interact with reactive radicals. In this context, GSH has been demonstrated to be an intracellular scavenger of free radials through enzymatic and nonenzymatic reactions (94, 95). The coupling between GSH metabolism and MT redox cycle would thus enable the antioxidant function of MT.

Perspectives

Ever since MT was discovered near half a century ago, studies of biologic function of this ubiquitous, small, metal-binding protein have progressed with uncertainty. Novel insights into the action of MT have been acquired recently through elucidation of the oxidoredutive properties of the metal-thiolate cluster of MT. Coordination of metals to the thiol group of cysteine residues of MT permits mobilization of metals through redox modulation of the ligand. The low redox potential of the thiolate cluster allows oxidation by a number of mild cellular oxidants that present constantly in the cell, with a dynamic change in redox state. Metal binding to MT is thermodynamically stable, but oxidation of the thiolate cluster leads to metal release and formation of MT disulfides or thionin (if all metals are released from MT). Reduction of MT-disulfide or thionin is possible when cellular reducing agents, such as GSH, along with selenium catalyst are available and MT is reconstituted in the presence of metals such as zinc under physiologic conditions. This process constitutes the MT redox cycle. The view of MT redox cycle would provide an alternate perspective on biologic function of MT, including homeostasis of essential metals, detoxification of toxic metals, and protection from oxidative stress. Compelling data from *in vivo* studies have to be obtained to elucidate the link between MT redox cycle and cellular metabolism and function.

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