Apo-Metallothionein Emerging as a Major Player in the Cellular Activities of Metallothionein

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Observations of apo-metallothionein (apo-MT) have been made under a variety of physiologic circumstances, including zinc deficiency in cell culture and in rodents, cellular induction of MT by dexamethasone with concurrent Zn deficiency, a variety of tumors under normal Zn conditions, MT induction by Zn and Bi citrate, induction of hepatic MT after tumor cell injection into nude mice, and overexpression of cardiac MT in MT transgenic mice. Experiments demonstrating both the lability of Zn and Cu bound to MT and the cellular stability of apo-MT are described to help rationalize the widespread presence of this metal-depleted species. Finally, comparative *in vitro* and cellular experiments examined the relative reactivity of Zn- and apo-MT with nitric oxide species, showing that apo-MT is much more reactive chemically and that in cells it may be a principal reactive species within the MT pool. Exp Biol Med 231:1528–1534, 2006

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Introduction

Nearly 50 years ago, Margoshes and Vallee reported the discovery of a mammalian Cd-binding protein, which they named *metallothionein* (1). In the following decades, physico-chemical studies showed it to have a unique amino acid sequence that is distinguished by its remarkable density of cysteinyl residues (2). These amino acids contribute to the sulfhydryl (SH) groups that are central to the 3-dimensional conformation and proposed functions of the holoprotein. They bind seven divalent metal ions and, in doing so, fold the protein into two domains, each of which contains a metal-thiolate cluster (3, 4). Metal ion binding reactions relate to one category of metallothionein functional properties; namely, participation in toxic and essential metal ion metabolism. In this capacity, metallothionein (MT) protects cells from the toxicity of excess metal ions (e.g., Cd^{2+}) and serves as a steady state or transient storage site for Zn^{2+} and Cu^{1+} during its intracellular trafficking (5–7).

The SH groups of the Zn-thiolate clusters also are chemically reactive sites with a variety of biologically relevant electrophiles and oxidants, including nitrogen mustards, Pt(II) complexes, and reactive oxygen species (8–13). The SH reactivity of Zn_7-MT is impressive when compared with other Zn proteins and probably results from the solvent accessibility of its metal-thiolate clusters as well as the concentration of SH groups in the protein that favor intramolecular reaction of multiple thiolates with reactive species (14–17). Nevertheless, the thiolates are considerably less reactive in Zn_7-MT than in metal-free apo-metallothionein (apo-MT) (18–20). Are there roles for apo-MT in the cellular biochemistry of the protein?

Historically, the significance of this question has been dismissed. Apo-MT is a random coil structure that is degraded by proteases *in vitro* much faster than metal-bound forms (21). Equating the apo-MT structure with denatured forms of other proteins that are rapidly biodegraded in cells, it has been assumed that apo-MT cannot exist for any length of time within the cell (22). However, nearly two decades ago we found that Zn-MT, which is constitutively expressed in Ehrlich tumor cells, was converted into apo-MT in Zn-deficient mice. This apo-MT remained 5 weeks after imposition of the Zn-deficient diet that brought Ehrlich cell proliferation to a halt (23). Similar results were observed in culture after Ehrlich cell exposure to a Zn-depleted medium (24). In culture, the biodegradation rate constant for the apo form was determined to be similar to that for the overall protein pool, showing that its lack of 3-dimensional structure did not consign the protein to an unusually rapid turnover rate (24). A peculiar property of both Zn-deficient cell populations was that MT was the only obviously labile pool of Zn under these conditions.

Following those observations, Pattanaik *et al.* showed

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that the metallothionein pool of a variety of tumors is both elevated in concentration and present in part or wholly as apo-MT (25). Subsequently, Maret *et al.* discovered that apo-MT constitutes a significant portion of the constitutive pool of MT in a number of rodent tissues (26). Thus, apo-MT or metal-unsaturated MT exists in many normal and transformed cells as a constitutively expressed species.

Methodology to Detect Apo-Metallothionein

Three methods have been used to reveal cellular apo-MT. The one that we have used is effectively a modification of the Cd-heme method for the quantification of total MT protein (27, 28). It is designed so that the metal and SH content of the native protein can be measured together with the total protein. Cell supernatant is divided in two, and one of the two groups is directly chromatographed over Sephadex G-75 (Amersham Biosciences, Uppsala, Sweden) to separate the MT fraction. The other group is exposed for 15 mins to Cd in order to saturate apo-MT metal-binding sites and displace bound Zn. Sephadex G-75 chromatography follows. Cd, Zn, and Cu (atomic absorption spectrophotometry) and SH (Ellman's assay) concentrations are determined on the fractions of each separation (14). A comparison of either the total metal or SH concentrations of the MT region of the two chromatographic profiles will reveal immediately whether apo-MT exists in the native sample. The procedure can be repeated in the presence of 2-mercaptoethanol to see whether oxidized apo-MT is present in the chromatographed samples.

Others have employed high-performance liquid chromatography (HPLC) and fluorometric means to isolate the apo-MT fraction of the MT pool (26, 29). In contrast to our method, neither of these methods assesses the metal content nor the ratio of metal occupied to metal-free sites in the protein.

Apo-Metallothionein: Means of Cellular Production

Previous experiments have demonstrated the presence of steady-state basal expression of apo-MT. In the sections below, we describe different conditions that result in elevated cellular apo-MT.

Bismuth Citrate. Exposure of animals to Bi subnitrate induces the synthesis of MT and has been used as a means to protect them from various electrophiles and oxidants. Thus, mice pretreated with Bi receive significant protection from the dose-limiting toxicities of *cis*-dichlorodiammine Pt(II) in the kidney and adriamycin/doxorubicin in the heart (30, 31). Because most studies had not characterized the metal status of the induced pool of MT, we examined the properties of LLC-PK_1 cells treated with a nontoxic concentration of 480 μM Bi citrate. Bi citrate was employed because it represents a solubilized form of Bi^{3+}.

A 24-hr incubation of cells with Bi citrate resulted in an upregulation of MT synthesis (Table 1). Nevertheless, in this cell line the MT pool was almost devoid of Bi and contained little Zn. Instead, the protein was mostly apo-MT, despite the fact that large concentrations of Bi were detected in the aggregate high–molecular weight (>20 kDa, HMW) and low–molecular weight (<5 kDa, LMW) fractions. Notably, the Zn content of the aggregate HMW Zn protein pool remained stable upon the introduction of the large concentration of unsaturated Zn binding sites in apo-MT, indicating that most cellular Zn binding sites are kinetically inert to reaction with apo-MT. In contrast, in another cell line, U373 glioblastoma, exposure to Bi citrate resulted in cells that had turned a yellow-green color. They contained an induced pool of MT that was largely saturated with Bi, according to Sephadex G-75 chromatography of the cytosol and Bi and an SH analysis of the column fractions. A spectrum of the isolated, greenish metallothionein fractions (Fig. 1) revealed a spectrum with shoulders at 270 nm and 320 nm, characteristic of Bi-MT.

The lack of MT-bound Zn in LLC-PK_1 cells exposed to Bi was surprising, since the external medium contained a full complement of Zn^{2+}. Nevertheless, apo-MT also has been detected in a number of cell lines grown in culture under similar conditions (25). Together, these results lead to the conclusion that the existence of a cellular thermodynamic sink for Zn is not sufficient to demand the transport of sufficient Zn from the extracellular medium to convert the pool into saturated Zn-MT.

Zinc. The lack of a tight coupling of Zn influx with MT synthesis is most dramatic in experiments examining the effect of an incubation of cells with elevated concentrations of Zn. Using LLC-PK_1 and TE671 cells, incubation with 40–80 μM Zn for 24 hrs resulted in the synthesis of large concentrations of MT that were 60%–70% saturated with Zn (Table 1). Extending the length of incubation to 72 hrs failed to enhance the degree of MT saturation with Zn. Thus, the lack of Zn saturation did not appear to result from a short-term rate limitation in Zn influx. These results are remarkable because they suggest that the presence of an abundant extracellular source of nutrient Zn is not necessarily sufficient to insure the generation of Zn-MT. One may infer that other conditions of elevated MT production under Zn-normal or Zn-deficient conditions might result in apo-MT. A further hypothesis is that the extent of Zn saturation of cellular MT depends not only on the intracellular dynamics of MT but also on the plasma membrane Zn transport characteristics.

Tumor Cell Injection in Mice. Zn-MT has been observed repeatedly in the liver of animals stimulated with a variety of stress agents (32). The induction of hepatic MT coincides with the shift of Zn out of the plasma, as described above. Tumor cells act as inflammatory host stressors and induce MT in the liver (33). Implantation of U373 glioblastoma cells into nude mice induces the synthesis of liver MT. Surprisingly, 2 days after implantation the hepatic MT pool was 30% apoprotein (Table 1). This result suggests

Table 1. Apo-Metallothionein Concentration in Cells under Different Conditions

Condition	Total metallothionein	Apo-metallothionein
Bi citrate: 480 μ*M* for 24 hrs		
LLC-PK$_1$ cells[a]	0.31 nmol/10^7 cells	0.27 nmol/10^7 cells
Zn[b]		
TE671 cells[b]: 24 hrs induction with 80 μ*M* Zn^{2+}	1.0 nmol/10^7 cells	0.40 nmol/10^7 cells
LLC-PK$_1$ cells[a]: 24 hrs induction with 40 μ*M* Zn^{2+}	1.3 nmol/10^7 cells	0.30 nmol/10^7 cells
Tumor		
Control nude mouse liver	12 nmol per g tissue	0 nmol per g tissue
Liver: 2-day induction with U373 tumor cells	19 nmol per g tissue	7 nmol per g tissue
Transgenic mouse heart	73 nmol per g tissue	26 nmol per g tissue
Dexamethasone		
U373 cells[c]: control	0.30 nmol/10^7 cells	0.20 nmol/10^7 cells
25 μ*M*: 4 hrs induction, Zn deficiency	0.73 nmol/10^7 cells	0.49 nmol/10^7 cells

[a] Culture conditions: Cells were grown in Medium 199 with 25 m*M* HEPES. This was supplemented with 3% fetal calf serum, 50,000 U penicillin/l, and 50 mg/l streptomycin. The cells were cultured at 37°C in a 5% carbon dioxide atmosphere. Metallothionein metal and metal-free binding sites (apo-MT) were determined as described in the section Methodology to Detect Apo-metallothionein.
[b] Culture conditions: Cells were grown in Dulbecco's modified Eagle's medium with 1 g/l glucose that was supplemented with 5% fetal calf serum, 50,000 U penicillin/l, and 50 mg/l streptomycin. Cultures were maintained in a 37°C incubator in a 5% carbon dioxide atmosphere. Metallothionein metal and metal-free binding sites (apo-MT) were determined as described in the section Methodology to Detect Apo-metallothionein.
[c] Culture conditions: The cells were grown as in footnote *b*, but supplemented instead with 10% fetal calf serum. Metallothionein metal and metal-free binding sites (apo-MT) were determined as described in the section Methodology to Detect Apo-Metallothionein.

that induced apo-MT will be found as a significant species in hepatic cells as part of the generalized host response to stress.

Transgenic Heart MT. Adriamycin/doxorubicin is a heavily used anticancer drug that elicits dose-limiting toxicity in the heart. Studies have shown that this severe side effect is suppressed in MT-transgenic mice with an elevated expression of cardiac MT (12). It has been proposed that reactive oxygen species generated by the redox-active drug are responsible for toxicity and that MT counteracts their deleterious effects through reaction of its SH groups with these oxygen species. We examined the speciation of MT in hearts from control FVB and MT-transgenic mice obtained from Y.J. Kang (University of Louisville, KY). Control mice contained no measurable MT.

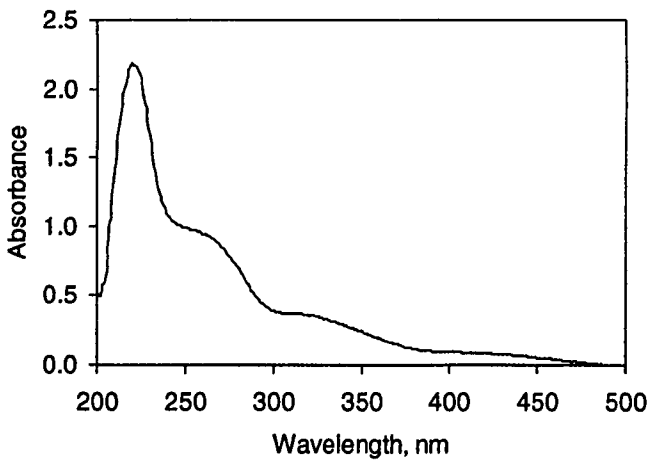

Figure 1. Spectrum of Bi-MT from the Sephadex G-75 fractionation of cytosol from U373 glioblastoma cells grown in culture in the presence of 500 μ*M* Bi as Bi citrate for 70 hrs. Culture conditions were as described in Table 1.

In comparison, the heart-specific, MT-transgenic mice contained 47 nmol/g Zn-MT and 26 nmol/g apo-MT in their heart muscle, fully 35% of the total MT pool (Table 1). The quantity of Zn-MT represents 63% of the total cytosolic Zn, demonstrating the huge enhancement in both MT production and Zn accumulation in the transgenic mouse heart. The finding of large concentrations of apo-MT in the heart muscle raises the question of the relative contributions of Zn-MT and apo-MT to the cardiac protection observed in the MT mouse.

Dexamethasone and Zn Deficiency. Based upon earlier work, it was hypothesized that Zn-MT could be converted into apo-MT by exposure to conditions of true Zn deficiency in the extracellular medium (24). An extension of this idea is to suggest that under Zn-deficient conditions successful induction of MT generates apo-MT. This condition may come into play in cells and tissues after a host stress response in which the reactive plasma Zn pool plummets upon onset of the stress (32). Dexamethasone (Dex), a glucocorticoidlike stress hormone, is a potent inducer of Zn-MT. In the absence of external Zn, Dex remains an efficient inducer, producing about two-thirds the amount of protein (apo-MT) that is formed under Zn-normal conditions (Zn-MT; Table 1).

TPEN. TPEN(N,N,N′N′-tetrakis(2-pyridylmethyl)-ethylene-diamine) is a multidentate metal chelating agent that has been used as a membrane-permeable reagent to lower labile intracellular Zn concentrations (34). Indeed, its effect has been likened to that of establishing conditions of intracellular Zn deficiency (35). After inducing the synthesis of Zn-MT in TE671 cells with Zn, the addition of 25 μ*M* TPEN for 30 mins markedly reduced the Zn content of the MT pool without clearly affecting HMW Zn (Fig. 2). The

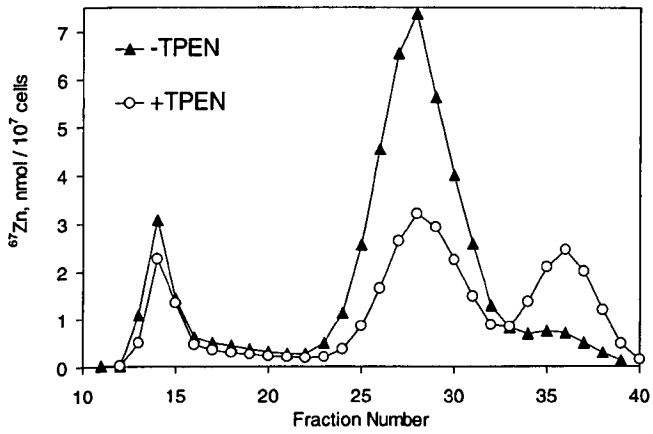

Figure 2. Sephadex G-75 gel filtration fractionation of LLC-PK$_1$ cultured pig kidney cells exposed to 40 *M* of $^{67}Zn^{2+}$ for 24 hrs followed by a 30-min incubation with or without 25 *M* TPEN. Isotopic ^{67}Zn was measured by ICPMS. Culture conditions were as described in Table 1.

observed increase in LMW Zn is thought to represent the presence of Zn-TPEN. In this model the rapid competition of TPEN for Zn bound to MT must involve the direct reaction of TPEN with Zn-MT. The process appears to be monophasic and bimolecular in nature, with a rate constant of 10 $M^{-1}\cdot sec^{-1}$. The selectivity of the cellular Zn-MT pool for reaction with this powerful chelating agent underscores the peculiar kinetic lability of Zn-MT in comparison with the inertness of the aggregate HMW Zn pool.

Cellular Metal Metabolism/Trafficking and Apo-MT

Zn-Deficient Conditions. Large intracellular concentrations of apo-MT can be achieved under a variety of conditions. Considering that apo-MT represents a moderately high-affinity Zn binding site ($K_d \sim 10^{-11}$ per Zn), the question arises as to how it is maintained in the metal-free state in the presence of a host of Zn proteins as well as an extracellular supply of Zn (36, 37). Conversely, how do other Zn proteins maintain their Zn? Several results bear on this question. An earlier experiment demonstrated the rapid loss of Zn from the basal MT pool in Ehrlich cells under Zn-deficient culture conditions (24). Zn moves out of the protein with a rate constant several times greater (0.7/hr) than that for MT protein turnover and general protein biodegradation (0.1/hr), which is consistent with the operation of a chemical mechanism of Zn liberation involving the native protein. We favor transfer of Zn out of Zn-MT by ligand substitution, not labilization, of the Zn-thiolate clusters by oxidation with oxidized glutathione as proposed by Maret because the latter reaction occurs on a much slower time scale *in vitro* (17, 38). Considering the similarity of the biodegradation rate constants for apo-MT and other cellular proteins, it is not surprising that apo-MT might be generated and observable upon induction by dexamethasone.

Zn-Normal Conditions. The kinetics observed under Zn-deficient conditions parallel those determined in the presence of Zn under steady-state conditions. We employed a dual Zn isotope method to follow the flux of Zn through the MT pool of TE671 cells (39). Cells were uniformly labeled with ^{67}Zn and then at Time 0 were shifted to an identical medium containing ^{66}Zn in place of ^{67}Zn. Subsequently, the cells took up ^{66}Zn commensurate with their requirements as they proliferated (doubling time, 16 hrs). Sephadex G-75 chromatography was used to separate the MT pool over time, and inductively coupled plasma mass spectrometry was used to assess quantitatively the MT concentrations of ^{66}Zn and ^{67}Zn. As shown in Figure 3, ^{66}Zn rapidly replaced MT-bound ^{67}Zn. Other measurements show that intracellular ^{66}Zn did not return to the external medium. Apparently, under these *demand* conditions for external Zn created by cell proliferation, MT acts as a fast kinetic compartment ($t_{1/2}$ = 2 hrs) that rapidly couples at least part of the influx of Zn with the need for Zn by newly synthesized cellular proteins. We hypothesize that the actual steady-state occupancy of the MT pool with Zn is a sensitive function of the rate of Zn transport into the cell and the rate of transfer of Zn from MT to other sites:

$$\text{Zn input into MT} \rightarrow \text{MT-Zn occupancy} \rightarrow \text{Zn output of MT.} \quad (1)$$

Accordingly, MT should not be considered a competitor for intracellular Zn but instead a contributor to the normal metabolic distribution of Zn to apo-Zn proteins.

This view is supported by a previous experiment that made a connection between the proliferation state of U373 cells and the Zn saturation state of MT (6). U373 cells normally contain a constitutive pool of apo-MT (6). Apo-MT is converted to Zn-MT upon inhibition of cell division with thymidine. Lifting the thymidine block restores the protein's metal-free status as the cells re-enter their proliferative state in which more Zn proteins need to be synthesized. In this manipulation, MT behaves like a labile intermediate or storage site in Zn trafficking, and MT-Zn occupancy depends on the cellular demand for Zn.

The other side of the pathway shown in Eq. 1 is the rate of net Zn transport into cells. If it does not match the rate of Zn transfer out of MT, the protein also will be unsaturated in Zn. The striking difference in the Zn content of MT after dexamethasone and Bi citrate induction in Zn-normal media may reflect differences in the response of plasma membrane Zn transporters to these two inducers (40). There is some indication that Dex upregulates Zn transport into rat hepatocytes (41). Perhaps, in contrast, Bi^{3+} has no impact or has a negative impact on transport such that Zn transport does not keep up with the expansion of the intracellular apo-MT pool.

Cu Excess Conditions. Encouraging the hypothesis that the rate and net direction of metal ion transport contribute to the resultant metal ion saturation state of MT is

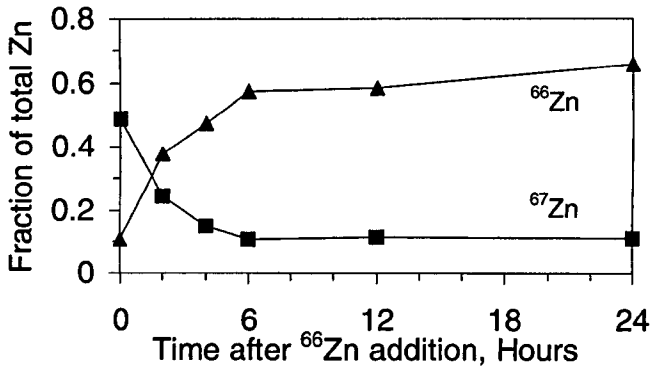

Figure 3. TE671 cells grown for longer than 6 months in media containing 8 μ*M* isotopically enriched ^{67}Zn were rinsed and transferred to media containing 8 μ*M* isotopically enriched ^{66}Zn. At t = 0, 2, 4, 6, 12, and 24 hrs after the media change, cell cytosols were analyzed by Sephadex G-75 gel filtration fractionation, followed by analysis using inductively coupled plasma mass spectroscopy (ICPMS). This technique allows the analysis of individual zinc isotope levels. Culture conditions were as described in Table 1.

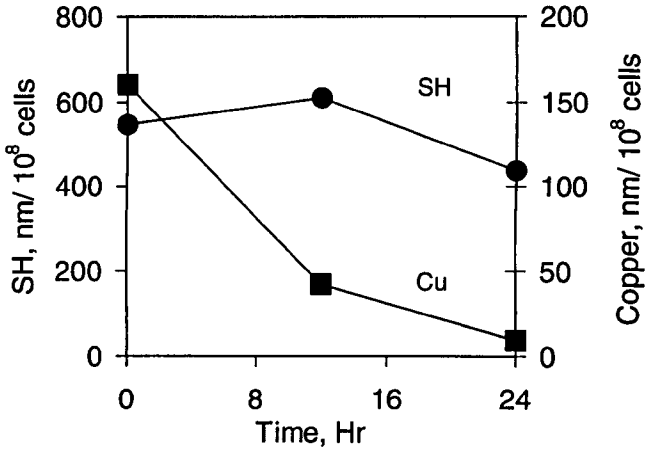

Figure 4. U373 glioblastoma cells were grown as described in Table 1 in media containing 50 μ*M* $CuCl_2$ for 24 hrs. At t = 0 the media was removed and replaced with fresh media containing no copper. At time points up to 24 hrs after the removal of the copper, Sephadex G-75 gel filtration columns were run on the cell cytosol. DNTB (5,5'-Dithiobis(2-nitrobenzoic acid)) analysis of the SH groups and atomic absorption analysis of the copper were performed on the MT fractions, and the data were plotted as nmol per 10^8 cells.

an experiment in which U373 cells were preinduced with 50 μ*M* Cu for 24 hrs to generate a large pool of cellular Cu-MT. Then, the cells were transferred into the normal medium in the absence of Cu. Within the next 12 hrs this reservoir of intracellular Cu was expelled to the medium as Cu-MT was converted to apo-MT according to Sephadex G-75 chromatography as well as Cu and SH analysis of the cell cytosol (Fig. 4). The prolonged retention of an elevated concentration of apo-MT after Cu had departed emphasizes that the export mechanism operated in conjunction with MT, not as a consequence of its degradation. In this case, we suggest that the Cu P-type ATPase and Cu chaperone, MNK/ccc2, serves as the active Cu exporter (42).

Functional Consequences of Apo-MT Formation for the Mechanism of Reaction of MT with Oxidants and Electrophiles

The identification of apo-MT in cells under the gamut of conditions from Zn deficient to Zn excessive and after induction of MT synthesis with a variety of inducers besides Zn leads to the hypothesis that apo-MT may be a common cellular species. The chemistry of reaction of oxidants and electrophiles with apo-MT and Zn-MT is expected to differ with respect to rate of reaction and, probably, in the nature of the observable reactions. For example, Zn-MT is kinetically inert to oxidation-reduction reaction with CrO_4^{2-}, whereas the SH groups of apo-MT react more rapidly with chromate than does GSH (20). Likewise, Zn-MT as a nucleophile is unreactive with iodoacetamide, unlike apo-MT, which reacts virtually within the time of mixing (18, 19). Therefore, we need to consider whether apo-MT is an important contributor to the redox and nucleophilic reactions usually attributed to Zn-MT.

MT Plus Nitric Oxide (NO). In an initial study of the relative roles of Zn-MT and apo-MT as cellular sites of reaction of electrophiles and oxidants, the reactivities of apo-MT and Zn-MT with NO species were examined *in vitro* and in cells. Under anaerobic conditions, NO is unreactive with Zn-MT (43). In contrast, when NO can be oxidized to NO_2 and N_2O_3, the Zn-thiolate clusters are slowly oxidized, probably to disulfides and, perhaps, higher valent sulfur species. The higher reactivity of apo-MT was shown by its rapid reaction with NO even under anaerobic conditions.

It was hypothesized that this differential reactivity would reflect itself in cells by a preferential reaction of diethylamine-nonoate (DEA-NO) with apo-MT. In order to test this idea, TE671 cells were induced for 24 hrs with 80 μ*M* Zn to make a combination of Zn-MT and apo-MT as summarized in Table 1. They were exposed aerobically for 1 hr to a nontoxic concentration of 300 μ*M* DEA-NO, and their cytosols were subjected to Sephadex G-75 chromatography to separate the aggregate protein (HMW), MT, and glutathione (GSH) pools of thiols and Zn. Upon exposure to DEA-NO, SH groups in each aggregate pool reacted similarly and extensively with HMW, GSH, and MT pools of SH groups as quantified in Table 2. Surprisingly, at least as many as 30% of the cellular thiols can be modified without acute toxicity. Clearly, neither the MT nor the glutathione pool displays preferential reactivity with the NO species in comparison with each other or with the HMW protein pool.

Table 2 also reveals that DEA-NO reacted exclusively with the apo-MT fraction of the MT pool in cells induced to make extra MT. The SH content of MT was depressed in response to exposure to DEA-NO without loss of Zn, signaling the reaction with apo-MT. Not only was Zn-MT unreactive in comparison with apoMT, it was unreactive in relation to much of the aggregate protein HMW band as well as glutathione. Future experiments will need to

Table 2. Zn and SH Concentrations in TE671 Cell Pools in the Presence and Absence of DEA-NO[a]

	HMW		MT		GSH	
	Zn	SH	Zn	SH	Zn	SH
+Zn,−DEA-NO	85	1280	41	200	22	440
+Zn,+DEA-NO	63	990	42	160	20	350

[a] DEA-NO concentration: 450 μ*M* for 1 hr. Zn and SH concentrations are expressed in nmol/10^8 cells.

harmonize these results with others that implicate the release of Zn from MT in the reaction of NO derived from *S*-nitrosocysteine (44, 45).

Conclusions

The apparently widespread presence of apo-MT in cells under basal and induced conditions makes it necessary to consider the possibility that among MT species apo-MT is preferentially reactive with oxidants and electrophiles. Initial experiments with NO are consistent with this hypothesis. The observation of apo-MT under diverse circumstances also requires a rethinking of Zn and Cu metabolism/trafficking in relation to MT. The MT pool does not act like a thermodyamic sink for these metals but, rather, acts more like an intermediate in their trafficking.

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