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Zinc Binding Ligands and Cellular Zinc Trafficking: Apo-metallothionein, Glutathione, TPEN, Proteomic Zinc, and Zn-Sp1

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Abstract

Many cell types contain metal-ion unsaturated metallothionein (MT). Considering the Zn^{2+} binding affinity of metallothionein, the existence of this species in the intracellular environment constitutes a substantial “thermodynamic sink.” Indeed, the mM concentration of glutathione may be thought of in the same way. In order to understand how apo-MT and the rest of the Zn-proteome manage to co-exist, experiments examined the in vitro reactivity of Zn-proteome with apo-MT, glutathione (GSH), and a series of common Zn^{2+} chelating agents including N,N,N',N'-(2-pyridylethyl) ethylenediammine (TPEN), EDTA, and [(2,2'-oxypropylene-dinitrilo]tetraacetic acid (EGTA). Less than 10% of Zn-proteome from U87 mg cells reacted with apo-MT or GSH. In contrast, each of the synthetic chelators was 2–3 times more reactive. TPEN, a cell permeant reagent, also reacted rapidly with both Zn-proteome and Zn-MT in LLC-PK₁ cells. Taking a specific zinc finger protein for further study, apo-MT, GSH, and TPEN inhibited the binding of Zn₃-Sp1 with its cognate DNA site (GC-1) in the sodium-glucose co-transporter promoter of mouse kidney. In contrast, preformation of Zn₃-Sp1-(GC-1) prevented reaction with apo-MT and GSH; TPEN remained active but at a higher concentration. Whereas, Zn₃-Sp1 is active in cells containing apo-MT and GSH, exposure of LLC-PK₁ cells to TPEN for 24 h largely inactivated its DNA binding activity. The results help to rationalize the steady state presence of cellular apo-MT in the midst of the many, diverse members of the Zn-proteome. They also show that TPEN is a robust intracellular chelator of proteomic Zn^{2+} .

Introduction

Zn^{2+} constitutes a catalytic or structural cofactor for thousands of metalloproteins [1-3]. Besides its role as a component of enzyme active sites it also provides critical structural stabilization to a multitude of proteins, including a multitude of zinc-finger transcription factors [3,4]. Despite its familiar role in protein structure and function, relatively little is known about Zn^{2+} trafficking that brings Zn^{2+} to such sites. Work in this area has largely focused on the identification of a variety of transporters that specifically facilitate the movement of Zn^{2+} into and out of cells and between subcellular compartments [5]. Thus, in contrast to the rapidly emerging understanding of the chaperone chemistry that specifically guides Cu^{1+} between the cell surface and its relative few protein binding sites, the intracellular reactions that result in the formation of specific Zn-protein complexes remain largely unknown [6].

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A prominent, enigmatic participant in Zn^{2+} metabolism is the small, thiol-rich protein, metallothionein (MT). MT's large stability constant at pH 7.4, 2×10^{11} per Zn^{2+} , suggests a role in cellular Zn^{2+} storage [7,8]. Yet, its facile metal ion exchange and ligand substitution reactions point toward a possible role in Zn^{2+} trafficking [9-11]. Beyond potential activities in Zn^{2+} metabolism, experimental evidence strongly supports the involvement of MT in reactions with toxic and therapeutic metals, electrophiles, and oxidants that protect cells from the deleterious effects of such reagents [12-14]. Considered from this perspective, the sulfhydryl reactivity of MT's 20 thiolate groups becomes of central interest.

The discovery that a variety of tumor and normal cells contain metal-unsaturated or apo-metalllothionein has complicated this picture [15,16]. The free SH groups of apo-MT react with a variety of reagents, including iodoacetamide, chromate, NO, and S-nitroso-compounds, much more rapidly than the thiolate groups of the Zn-thiolate clusters of $\text{Zn}_7\text{-MT}$ [17-19]. Moreover, the presence of apo-MT places what might be termed a thermodynamic sink for Zn^{2+} in the midst of the hundreds of Zn-proteins that populate the cell.

How is the metal-unsaturated state of cellular MT maintained in the midst of the array of other Zn-proteins, the Zn-proteome, and an extracellular source of nutrient Zn^{2+} ? Conversely, how do Zn-proteins retain their integrity in the presence of apo-MT's high affinity Zn^{2+} binding sites? These are the principal questions addressed in this study.

The general process that we have examined is the ligand substitution reaction between a cellular source of Zn^{2+} and a competing Zn^{2+} binding ligand, such as apo-MT:

As the interactions between apo-MT, a potent Zn^{2+} chelator, and the rest of the intracellular Zn-proteome are considered, one also needs to include its companion thiol-containing peptide, glutathione (GSH), into the analysis. GSH binds Zn^{2+} less tightly than apo-MT but exists in cells in mM concentration [20,21]. For comparison, we have also investigated the intracellular behavior of a commonly used cell membrane permeant Zn^{2+} chelator, N,N,N',N'-(2-pyridylethyl)ethylenediamine (TPEN) and selected aminocarboxylate chelating ligands [22].

Experimental

Materials

EDTA (Fisher Scientific), EGTA (Sigma-Aldrich) were reagent grade. TPEN was obtained from Sigma-Aldrich- and was the highest grade available. Human recombinant $\text{Zn}_3\text{-Sp1}$ was a product of Promega (Madison, WI) and its antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GC-1 oligonucleotide (HPLC purified) was obtained from Integrated DNA Technologies. All other chemicals were chemicals were reagent grade or highest purity available.

Cell Culture

U-87 mg human glioblastoma-astrocytoma cells (ATCC number HTB-14) were grown in Eagle's Minimal Essential Medium with non-essential amino acids (Sigma M 0643) supplemented with 6% fetal calf's serum. The cells were maintained in a 37° C incubator with a 6% CO_2 atmosphere. LL-CPK₁ pig kidney cells (ATCC number CL-101) were grown in Medium 199, HEPES modification, (Sigma M 2520) supplemented with 3.5% fetal calf's serum and also kept in a 37° C incubator in a 6% CO_2 atmosphere. Media zinc levels are almost completely provided by the fetal calf's serum and varied from 1.8–3.0 μM depending on the serum concentration.

Cellular Apo-MT Sites Analysis

For in vitro experiments, cells were lysed and sonicated. After centrifugation at 20,000 rpm for 20 minutes the supernatant was divided in 2 aliquots and one aliquot was incubated with Cd (PYR)₂ for 1 min [23]. Then, both supernatants were chromatographed on Sephadex G-75 column to resolve the metallothionein peak. The resultant fractions were analyzed for Zn²⁺ and Cd²⁺ by atomic absorption spectrophotometry. For in vivo analysis, the cells were treated with Cd(PYR)₂ mixed in Dulbecco's Phosphate Buffered Saline (DPBS) for 30 min. Then, control and Cd(PYR)₂ treated cells were lysed and the supernatants chromatographed as above. Table 1 describes the analysis of the results.

Preparation of rabbit liver MT 2

The metallothionein used in these experiments was purified using gel filtration liquid chromatography followed by DEAE anion exchange separation of the two isomers, MT 1 and MT 2 [24]. Briefly, male New Zealand white rabbits were given 8 daily 2.0 mL subcutaneous injections of 0.15 M ZnSO₄ to induce Zn₇-MT. Animals were sacrificed 24 h after the last injection and their livers removed, quickly weighed and frozen.

One hundred grams of the liver (approximately one liver) were homogenized in a Waring Blender with 200 mL of cold, degassed homogenizing buffer, 0.25 M sucrose in 5 mM Tris Cl, pH 7.6, with 10 mM 2-mercaptoethanol, and 0.5 ml of Phenyl-Methyl-Sulfonyl Fluoride-saturated ethanol. The suspension was centrifuged 10,000 g × 20 minutes in a high-speed centrifuge. The supernatant was further centrifuged at 100,000 g in a Beckman ultracentrifuge for 60 min. All procedures were performed at 4° C.

The ultracentrifuge supernatant was applied to a cold 12 × 85 cm Sephadex G-75 gel filtration column previously equilibrated with degassed 5 mM Tris Cl, pH 8.0, with 5 mM 2-mercaptoethanol. After most of the hemoglobin had passed through the column, fractions were collected and assayed for zinc. The fractions containing zinc were pooled, about 1.0–1.5 L, and applied to a HPLC DEAE column, BioRad 21.5 mm × 15 cm semipreparative DEAE 5PW column, washed with 5 mM Tris-Cl, pH 7.4 until the mercaptoethanol had eluted. The MT isoforms were eluted in a 400 mL Tris gradient from 5 to 300 mM. Fractions were collected and the conductivity and zinc content analyzed. Tubes in the conductivity range of 1–4 milliSiemens containing zinc were of interest. Two peaks corresponding to MT 1 (first) and MT 2 (second) were identified and the two isoforms stored at 4° C until needed. For the current studies, we used MT 2.

Apo-MT preparation

Apo-MT was prepared by adding concentrated HCl to the rabbit Zn-MT solution to a final concentration of 1.2 N HCl, giving a pH of approximately 3. The mixture was incubated for 15 min at room temperature and chromatographed over Sephadex G-15 column at pH 2 with 0.01 N HCl as elution buffer. Apo-MT was identified in the eluate by its absorbance at 220 nm. The concentration of apo-MT was determined using the colorimetric DTNB assay for sulfhydryl groups and removal of Zn²⁺ was checked by atomic absorption spectrophotometer. The product apo-protein was stored at 4° C under acidic and anaerobic conditions.

Metal Analysis

Metal analysis was done using atomic absorption spectrophotometry (GBC 904AA). Readings were standardized before each set with R² values > 99%.

Sulfhydryl Group Analysis

DTNB Analysis: 10mM DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)] was prepared in water and adjusted to pH=6.5 with Trizma base (Sigma). For sulfhydryl measurements, enough reagent was added to the sample in order to make a final concentration of 1mM DTNB. The solution was allowed to react for 1 hour (except in apoMT where no incubation time is required) and the absorbance was measured at 412 nm. The sulfhydryl content was measured directly from absorbance (Extinction coefficient = $13600 \text{ M}^{-1}\text{cm}^{-1}$).

Preparation of Zn-proteome

U87 mg, glioblastoma-astrocytoma, cells were removed from culture plates and the membranes were lysed by sonication. The lysate was centrifuged at 20,000 rpm and the supernatant was chromatographed over Sephadex G-75 column (0.7cm $\times$ 80 cm). From fraction number 10–20, the seven highest Zn containing fractions, were pooled and filtered. The pool was reconcentrated using Amicon ultra filtration devices with a 30 KDa molecular weight cutoff. The final Zn content was checked again using atomic absorption spectrophotometry and the protein was stored at 4° C and used within 24 h.

Electrophoretic Mobility Shift Assay

Recombinant human Zn₃-Sp1 (rhSp1) was purchased from Promega. Reagents from a DIG Gel Shift Kit, 2nd Generation (Roche) were used to label the oligonucleotide probe, to perform binding assays, and for detection by chemiluminescence. rhSp1 (~0.05 μM , 0.0011 nmol per reaction) was either untreated or incubated for 15 minutes at room temperature with increasing concentrations of apoMT, GSH, and TPEN. Then, 0.08 pmoles of labeled probe (GC rich GC-1 binding site for Sp1 in the sodium-glucose co-transporter promoter in mouse kidney) were added and binding reactions were carried out for an additional 15 minutes at room temperature [25]. Reaction mixtures were then separated on 6% non-denaturing poly acrylamide gels (Invitrogen) and blotted to a positively charged nylon membrane [25]. After incubating the blot with anti-DIG-Alkaline Phosphatase antibody, Phosphorescence substrate (CSPD-Roche) was added and the chemiluminescent bands were detected and quantified using a Kodak Image station. Quantified results represent the average of 2 experiments.

Results

Cellular Zn²⁺ Pools Measured by Gel Filtration Chromatography

Cytosol from cultured human U87 mg cells was fractionated into three pools of Zn²⁺, high molecular weight protein bound Zn²⁺, metallothionein associated Zn²⁺, and low molecular weight Zn²⁺ (Figure 1). The high molecular weight aggregate includes the complete collection of Zn²⁺ proteins minus the contribution from Zn-MT and for the purposes of this study is called Zn-proteome. The low molecular weight (LMW) pool migrates through Sephadex G-75 with a molecular volume slightly larger than GSH. This behavior indicated that low molecular weight Zn²⁺ exists in a ligand-bound form not as free Zn²⁺. This Zn²⁺ pool is routinely observed in gel filtration chromatograms of cytosol from a variety of cultured cells. Its properties will be described elsewhere.

Measurement of Intracellular Metal-unsaturated MT: Method improvement

Two methods have been employed to measure metal ion free sites in cellular MT. In the first, using gel filtration chromatography to isolate metallothionein, the metal ion contents of MT in native cell lysate are compared with those of lysate preincubated with Cd²⁺ in order to bind unoccupied metal ion binding sites as well as to displace Zn²⁺ bound to the protein [15]. The difference represents metal ion free sites. Using the alternative method, lysate is reacted with a thiol-specific thiol fluorophore that binds to free SH groups in MT and other proteins but not

to thiolate groups bound within metallothionein's Zn-thiolate clusters [16]. Then, the fluorophore-modified MT fraction is isolated and its fluorescence determined as a measure of free Zn^{2+} sites. Both procedures require cellular lysis and fractionation before analytical measurements are made, raising the possibility of Zn^{2+} reorganization during cell disruption.

We have improved the Cd^{2+} based assay by carrying out the reaction between MT and Cd^{2+} within the intact cell prior to cell lysis. The method involves the use of the Zn^{2+} ionophore, pyrithione (PYR), to deliver Cd^{2+} rapidly into cells [23]. There, $\text{Cd}(\text{PYR})_2$ readily reacts with apo-MT to generate $\text{Cd}_7\text{-MT}$. Once formed, the cells are broken and the MT protein isolated by gel filtration. Results from a typical experiment are shown in Figure 2. U87 mg cells were incubated with $\text{Cd}(\text{PYR})_2$ for 15 min, rinsed, lysed, and quickly chromatographed over Sephadex G-75 at room temperature. The Zn^{2+} and Cd^{2+} contents of Zn-proteome, MT, and LMW peaks were quantified and compared with values obtained from untreated cells. Table 1 shows the comparison as well as results from U87 mg cells in which apo-MT was reacted with Cd^{2+} after preparation of cell lysate. According to the two methods, about 30–35% of the MT fraction was unsaturated with Zn^{2+} or other metal ions. These findings provide an improvement on our method to detect cellular metal ion unsaturation in MT as well as bolster the validity of our earlier procedure to measure apo-MT.

Reaction of Zn-Proteome with Apo-MT and GSH

U87 mg cells have been investigated as an example of cells that contain a large constitutive pool of MT and within the pool a significant fraction of unsaturated binding sites. We isolated the Zn-proteome from U87 mg cells (peak I, Figure 1) and reacted it with apo-MT 2, prepared from rabbit liver, and then analyzed the extent of reaction with gel filtration chromatography (Figure 3). Concentrations of apo-MT Zn^{2+} sites twice that of the Zn^{2+} in each Zn-proteome sample were incubated for 30–45 min at 25° C under either aerobic or anaerobic conditions and similarly chromatographed under these conditions. According to Table 2, the comparative zinc mass balance between control and reaction samples was in excellent agreement. Generally, SH analysis of the isolated fractions showed the same congruence (data not shown). An average of 5% of the initial Zn-proteome shifted into the MT peak during these reactions. Similarly, 2 mM GSH sequestered 10% of the Zn^{2+} from the proteome pool under these reaction conditions (Table 2). These results demonstrate that little of the Zn-proteome is accessible to apo-MT or GSH and are qualitatively consistent with the repeated observation that apo-MT and GSH co-exist with Zn-proteome in a variety of cells.

Reaction of Zn-Proteome with Synthetic Chelating Agents

In the experiments above, apo-MT represents a high affinity Zn^{2+} chelating ligand; GSH is a weaker binding ligand despite its 2 mM concentration. The fact that neither reagent was a robust competitor for Zn^{2+} brings forth the question: Is the observed lack of reactivity a kinetic or thermodynamic phenomenon? In order to probe this question, Zn-proteome was incubated with a series of Zn^{2+} chelating agents ranging in equilibrium affinity for Zn^{2+} and the extent of ligand substitution measured as above. TPEN was chosen because it is commonly used as a cell permeant chelator of Zn^{2+} [22,26]. Little is known about its reactivity with intracellular Zn^{2+} pools. In addition, to fill in the range of ligands with varying apparent stability constants for Zn^{2+} at pH 7.4, the aminocarboxylate ligands, EDTA and EGTA were included as well [27-29].

Under the reaction conditions used with apo-MT and GSH, TPEN rapidly extracted 22–38% of the Zn^{2+} from Zn-proteome (Table 2); EDTA removed 32% (Figure 4, Table 2). EGTA, the weakest of these chelating agents, garnered 34%. According to these results, a significant fraction of the Zn-proteome is readily available for ligand substitution with these reagents. The data further suggest that the relative lack of reactivity of apo-MT and GSH results from

unfavorable kinetics in their interactions with Zn-proteome. Thus, EGTA is a much more robust competitor for proteomic Zn^{2+} than apo-MT yet binds Zn^{2+} with an apparent stability constant 2 orders of magnitude less than apo-MT.

Cellular Reactivity of Zn-Proteome and Zn-MT with TPEN

Cellular correlates of the in vitro reactivity of TPEN with the Zn-proteome and Zn-MT have been sought. TPEN was incubated with LL-CPK₁ cells, cell extracts were prepared, and the Zn^{2+} gel filtration profiles of treated vs. control cells compared. In one experiment, LLC-PK₁ cells were induced to express metallothionein (24 h incubation with 40 μM Zn^{2+}). Then, they were exposed to 100 μM TPEN for 10 min. According to gel filtration chromatography (Figure 5), the Zn-MT pool lost 83% of its Zn^{2+} and the Zn-proteome lost 28% during the short incubation time. The large increase in low molecular weight Zn^{2+} represents Zn-TPEN. The total Zn^{2+} recovery for the two samples agreed within 10%. In a second experiment involving control, uninduced cells, exposure to 25 μM TPEN for 30 min resulted in a 34% decline in Zn-proteome Zn^{2+} and a 50% decline in the small peak of Zn-MT. The two results mirror the in vitro findings that TPEN reacts with both Zn-proteome and Zn₇-MT.

Reaction of Specific Zn-protein, Zn₃-Sp1, with Apo-MT, GSH, and TPEN

A gross pattern of unreactivity of apo-MT and GSH with the Zn-proteome has been established. We were interested in the generality of this conclusion and have begun to extend this study to specific Zn-proteins. Zinc finger transcription factors with a C₂H₂ set of amino acid side chain ligands for Zn^{2+} are the most common type of Zn-protein in eukaryotic cells [3]. Among them, Zn₃-Sp1 is a 3-finger structure that participates in the transcriptional expression of many proteins [31]. Moreover, the Zn^{2+} stability constant of 1.7×10^9 for one of its fingers suggests that it can exchange Zn^{2+} with apo-MT [32]. Indeed, an early study confirmed that incubation of Zn₃-Sp1 with apo-MT inactivates Sp1 toward binding to a cognate DNA binding site [33].

We examined the integrity of human recombinant Zn₃-Sp1 in the presence of apo-MT, GSH, and TPEN, using the electrophoretic mobility shift assay (EMSA). The EMSA technique measures the capacity of control and treated Sp1 to bind to a cognate DNA binding site, in this case one of the Sp1 recognition sequences (GC-1) in the sodium glucose co-transporter 1 gene of mouse kidney [25]. Zn-finger mini-domains depend upon structural Zn^{2+} to establish their correct DNA-binding conformation [4,34]. Thus, the loss of DNA binding activity resulting from reaction with competing Zn^{2+} binding ligands can be attributed to peptide unfolding in the absence of Zn^{2+} . Since, in general, Zn₃-Sp1 can exist as the free protein or as protein bound to cognate DNA sequences, we tested both for their reactivity with competing ligands.

Figure 6 summarizes a typical series of reactions of Zn₃-Sp1 with apo-MT. Unbound GC-1 DNA ran to the bottom of the gel during electrophoresis; the Zn₃-Sp1-DNA adduct shown in the figure displayed a much retarded migration. Incubation of the free protein with apo-MT for 30 min followed by electrophoresis resulted in an apo-MT concentration dependent reduction in specific Zn₃-Sp1-(GC-1) adduct with 20% of control protein-DNA adduct observed after reaction with 30 μM Zn^{2+} binding sites. In contrast, pre-formation of the Zn₃-Sp1-(GC-1) adduct completely protected the Zn^{2+} sites from undergoing ligand substitution over the same concentration range of apo-MT.

Similarly, GSH reaction with Zn₃-Sp1 substantially inhibited its subsequent binding to GC-1 at the physiologically relevant concentration of 2 mM GSH (70% inhibition) as well as at lower concentrations. Nevertheless, as with apo-MT, GSH was ineffective against the Zn₃-Sp1-(GC-1) complex (Figure 7). Thus, both apo-MT and GSH displayed some reactivity toward Zn₃-Sp1, but neither was effective against Zn₃-Sp1's functionally active, DNA-bound form.

The pattern with TPEN was qualitatively similar (Figure 8). However, TPEN was a significantly more potent competitor for Zn₃-Sp1-bound Zn²⁺ in the absence as well as presence of DNA. Specifically, in order to reduce Zn₃-Sp1's capacity to bind to GC-1 by 80%, 30 μM Zn²⁺ binding sites in apo-MT were required compared with only 0.5 μM TPEN. Furthermore, when confronted with the Zn₃-Sp1-(GC-1) adduct, reductions in protein-DNA complex were observable at 10 μM TPEN.

Cellular Reaction of Zn₃-Sp1 with Apo-MT, GSH, and TPEN

In order to determine whether the findings about the reactivity of Zn₃-Sp1 with apo-MT and TPEN extend to intact cells, EMSA experiments were conducted on extracts of control U-87 mg cells that contain a substantial concentration of apo-MT (Table 1) as well as GSH. Despite the presence of these endogenous Zn²⁺ chelators, Zn-Sp1 was present in abundance in these cells.

LLCPK₁ cells also contain Zn₃-Sp1 as measured by the EMSA method and confirmed by including an Zn₃-Sp1-specific antibody in the EMSA reaction. The antibody supershifted some of the Zn₃-Sp1-(GC-1) band, indicating that the protein component of the band was, indeed, Zn₃Sp1 (Figure 9). Then cells were exposed to 100 μM TPEN for 30 min or 30 μM chelator for 24 h so that viability was not compromised. EMSA analysis showed that the brief treatment did not lower functional Zn₃-Sp1 but the 24 incubation was highly effective at 30 μM TPEN. Parallel western analysis showed that the same amount of Sp1 protein existed in control and TPEN-treated cells. Thus, the TPEN effect resulted in impaired DNA binding not loss of protein.

Discussion

Metallothionein has occupied the interests of numerous scientists for 50 years. First recognized for its singular capacity to bind intracellular Cd²⁺, attention broadened to include its interaction with other toxic metal ions, its participation in Zn²⁺ and Cu metabolism, and finally the role of its manifold sulfhydryl groups in electrophile and oxidant metabolism [5,12-14]. From a chemical standpoint, the properties of its unique metal ion-thiolate clusters continue to be the subject of investigation [35,36]. Its apparent multifunctionality, symbolized by the pleiotropic nature of the inducers of the protein, has left the protein without a consensus of well established roles within the cell [37].

Complicating this picture has been the unfolding story that under many physiological and pathological conditions, the steady-state MT protein pool includes metal-unsaturated sites, which we call apo-MT. Thus, in a variety of tumors a large fraction of metallothionein exists as apo-MT [15]. The apo-protein has been detected widely among normal tissues under basal expression conditions [16]. Similarly, in non-transformed cultured cells the protein may be unsaturated with Zn²⁺ and remain so when it is further induced by hormones or metal ions including Zn²⁺, itself [19]. This emerging generality probably applies to transgenically expressed metallothionein as well [19].

A question related to the identification of cellular apo-MT has centered on methods used to detect the metal-ion unsaturated form. Of necessity, they begin by disrupting cells to obtain an extract for analysis [15,16]. The possibility exists that Zn²⁺ might reorganize in relation to the MT pool when cells are broken. Previously, our method utilized Cd²⁺ to quantitatively tag metal ion-free sites in MT as described above. In a new modification, we treated cells directly with a permeable form of Cd²⁺, Cd(PYR)₂, and, thus, moved the reaction of Cd²⁺ with apo-MT from the cell extract into the undisturbed cells. According to Table 1, similar results are obtained with the original and modified methods. Besides validating the earlier procedure, the

new method provides a simpler way to address this question because it avoids the issue of thiol-oxidation that must always be considered with apo-MT in vitro.

In the past, apo-MT has been considered to be an unstructured polypeptide that gains its 3-dimensional structure upon formation of two metal ion-thiolate clusters in its α - and β -domains. In the absence of metal ion-thiolate bonding, its sulfhydryl groups are much more reactive [17,18]. Indeed, in one study, cellular reaction of the NO donor, DEA-NO, within the MT pool occurs almost exclusively with apo-MT [19]. Thus, the steady-state presence of metal ion unsaturated MT in cells expands the understanding of the reactivity of the metallothionein pool toward electrophiles and oxidants.

Nevertheless, U87 mg cells contain a significant fraction of apo-MT in their metallothionein pool. In effect, that fact indicates that both the high molecular weight fraction of Zn-proteins (Figure 1) or the Zn-proteome as well as the low molecular weight species of Zn (Zn-LMW) can co-exist with apo-MT. In turn, this implies that Zn^{2+} associated with Zn-proteome and Zn-LMW is largely kinetically inert to ligand substitution with apo-MT. In vitro experiments confirmed this conclusion. Incubation of the Zn-proteome with an excess of apo-MT for extended periods of time resulted in about 5% Zn^{2+} exchange into MT (Table 2).

A similar picture emerged from the experiments testing the reactivity of a large concentration of GSH with Zn-proteome (Table 2). These results show that the Zn-proteome is largely insensitive to the presence of the apo-MT and GSH pools. In offering this conclusion, it is recognized that individual Zn-proteins may change their Zn^{2+} occupancy in the presence of apo-MT while the overall Zn^{2+} status of the Zn-proteome appears unchanged. This possibility is discussed below.

The kinetic inertness of apo-MT with most of the Zn-proteome is surprising because of the generally held view that this protein serves the cell as a particularly robust metal ion chelator. The singularity of this finding is underscored by the results of experiments on the reactivity of Zn-proteome with other relevant chelating agents. Zn-proteome reacts rapidly with EDTA and EGTA, multidentate amine-carboxylate ligands, and TPEN, a cell-permeant ligand that is commonly used to chelate intracellular Zn^{2+} , under the same conditions that fail to yield reaction with apo-MT (Figure 4 and Table 2).

The apparent stability constant at pH 7.4 for each ligand tested with Zn-proteome is listed in Table 2. The lack of reactivity of apo-MT was striking because it binds Zn^{2+} with a log stability constant at pH 7.4 of 11.3, more than 100 times larger than EGTA. This finding makes clear that the relative lack of reactivity of apo-MT with Zn-proteome is a kinetic phenomenon. It is also noted that TPEN, EDTA, and EGTA successfully compete for similar fractions of Zn-proteome despite their 7 order of magnitude range in affinity for Zn^{2+} at pH 7.4.

From these results, one is left with a complicated view of the thermodynamic and kinetic stability of the Zn-proteome. If cells contain little or no unbound Zn^{2+} as is claimed, then on equilibrium grounds, it might be supposed that at least some Zn-proteins with modest stability constants for Zn^{2+} should undergo noticeable Zn^{2+} dissociation:

However, the apparent retention of protein bound Zn^{2+} , argues that there are substantial kinetic barriers to Zn^{2+} dissociation. These barriers must also extend to ligand substitution reactions with such competitors as apo-MT and GSH in order to leave the Zn-proteome intact. The results reported here support this view.

The general lack of reactivity of Zn-proteome with apo-MT does not exclude the possibility that individual Zn-proteins can undergo reaction. For example, an earlier study demonstrated that the reaction of the Zn-finger transcription factor, transcription factor IIIA, with apo-MT inactivated it toward specific DNA binding [30]. The present experiments confirm this finding and, in addition, show that GSH also competes successfully for Zn²⁺ bound to Sp1 under relevant cellular conditions (Figures 6 and 7). In unpublished experiments, we have also observed that GSH inactivates TFIIIA toward binding to cognate DNA.

Further inquiry into conditions that modulate Zn₃-Sp1 reactivity with apo-MT and GSH determined that pre-formation of the Zn₃-Sp1-(GC-1) adduct protected against reaction with apo-MT and GSH (Figures 6 and 7). Thus, the cellular integrity of Zn₃-Sp1 in the presence of apo-MT and GSH might be due to its existence as Zn₃-Sp1-DNA complexes. The protection afforded Zn₃-Sp1 through binding to a specific DNA sequence recalls a similar result with transcription factor IIIA and suggests that Zn-finger transcription factor-cognate DNA adducts might generally be kinetically inert to reaction with apo-MT [38]. The fact that the order of addition of reactants changes the outcome of the reactions points to kinetic factors as the basis for the lack of reactivity of Zn₃-Sp1-(GC-1) with apo-MT and GSH.

Cellular experiments revealed the presence of Zn₃-Sp1 in U87 mg cells that also contain a relatively large concentration of apo-MT. Their co-existence might imply that all of the endogenous Zn₃-Sp1 is bound to cognate DNA binding sites, rendering it unreactive with apo-MT. Alternatively, it is possible that the two proteins are isolated from one another by compartmentalization, Zn₃-Sp1 in the nucleus and apo-MT in the cytoplasm. In support of this possibility, numerous publications report cytoplasmic or nuclear localization of immunoreactive MT in a variety of tissues and cell types as well as conditions that foster a shift in its location between these compartments [39-44]. Because antibodies to MT generally detect both metal-bound and apo-MT, such studies do not provide needed information on the intracellular location of metal ion unsaturated MT. In order to better understand the co-existence of Zn²⁺ containing proteins such as Zn₃-Sp1, means to image apo-MT will need to be developed.

The steady-state presence of apo-MT in cells seems to defy the concept that unstructured peptides rapidly undergo biodegradation. The one measurement of the biodegradation rate for apo-MT done in transiently Zn-deficient Ehrlich cells revealed a first order process characterized by a rate constant of 0.07 h⁻¹ [10]. This was only 50% larger than the average rate constant of the cell cytosolic proteome. Recent theoretical calculations suggest that apo-MT retains its overall dumb-bell-like structure when metal ions are removed and, thus, that the demetallated protein has a 3-dimensional structure [45]. If this is so, apo-MT might be anticipated to degrade at a rate similar to those of other cellular proteins. In support of this view, an early study on apo-MT revealed that the metal-free protein moves like the holo-protein during gel filtration chromatography, consistent with its existence as a folded structure not as an unstructured polypeptide that would migrate as a larger molecule [46].

TPEN, like apo-MT and GSH, also competes for Zn²⁺ bound to free Zn₃-Sp1 and does so, but less efficiently with Zn-Sp1-(GC-1). This reaction may be significant in vivo since cellular exposure to TPEN also substantially reduced the concentration of endogenous Zn₃-Sp1 that can interact with the GC-1 probe (Figure 9). This last result suggests that cellular Zn₃-Sp1 and, perhaps other Zn-finger proteins are accessible to reaction with exogenous Zn²⁺ chelating agents. Nevertheless, the difference in reactivity of TPEN with intracellular Zn₃-Sp1 in comparison with apo-MT and GSH underscores the peculiar capacity of these two cellular Zn²⁺ binding ligands to co-exist with Zn-proteome.

The results of this study reveal the widespread reactivity of the Zn-proteome with TPEN. Application of this Zn²⁺ chelating agent has been employed to induce functional zinc

deficiency and demonstrate the role of intracellular Zn^{2+} in various processes such as apoptosis, oxidant stress, properties of neuronal synaptic transmission, regulation of transcription factors such as NF-kappa β , etc. [47-51]. The assumption seems to be that TPEN sequesters “chelatable” or “accessible” Zn^{2+} , defined vaguely as free or loosely bound Zn^{2+} ; but the actual impact of TPEN on Zn-proteome has not previously been measured. Present results demonstrate that TPEN's powerful ligand array readily and rapidly extracts a significant fraction of the Zn^{2+} associated with Zn-proteome in vitro and in cells. That being so, one can hypothesize that any or all of the functional effects caused by TPEN are due to non-specific Zn-protein inactivation not to sequestration of trafficking Zn^{2+} that might mimic true extracellular Zn^{2+} deficiency.

Lastly, the chemical issues related to steady state presence of apo-MT may be extended to cells exposed to Cd^{2+} , in which most of the Cd^{2+} becomes sequestered in MT. Earlier papers have addressed the question whether apo-MT might compete for Cd^{2+} that becomes bound to proteins in place of Zn^{2+} . Both Cd-TFIIIA and Cd-carbonic anhydrase readily transfer Cd^{2+} to apo-MT [38,52]. Indeed, the latter reaction occurs much more rapidly than the ligand substitution reaction between Cd-CA and EDTA. It was suggested that apo-MT and particularly the last 4 C-terminal cysteinyl thiolate groups readily react with Cd-substituted proteins as part of the mechanism to gather Cd^{2+} into the MT protein. If that is so, then further studies will be needed to understand the factors that make the Zn-proteome largely resistant to reaction with apo-MT but leave the related Cd-proteome susceptible to reaction.

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Abbreviations

Apo-MT, metal-ion unsaturated metallothionein that represents either completely metal-ion free protein or metal-ion depleted domains
 CA, carbonic anhydrase
 DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid)
 EGTA, [(2,2'-oxypropylene-dinitrilo]tetraacetic acid
 EMSA, electrophoretic mobility shift assay
 GSH, glutathione
 HMW proteins, high molecular weight proteins, contains the Zn-proteome
 MT, metallothionein
 PYR, pyrithione
 rhSp1, human recombinant Zn_3 -Sp1
 TPEN, N,N,N',N'-(2-pyridylethyl)ethylenediamine
 TFIIIA, transcription factor IIIA
 Zn-proteome, all of the Zn-proteins in the HMW fraction of gel filtered cytosol
 Zn-MT, MT protein containing Zn^{2+} but also unsaturated metal ion binding sites
 Zn₇-MT, MT protein with 7 bound Zn^{2+} ions
 Zn₃-Sp1, Sp1 protein with 3 bound Zn^{2+} ions

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Figure 1.
Sephadex G-75 gel filtration chromatography of cytosol from control U87 mg cells.

Figure 2.

Sephadex G-75 gel filtration chromatography of Zn-proteome from U87 mg cells exposed to Cd(PYR)₂. (A) Control U87 mg cells (B) Cd(PYR)₂ treated cells. Conditions of reaction: 2×10^8 cells incubated with $3 \mu\text{M}$ Cd²⁺ and $3 \mu\text{M}$ PYR for 30 min in DPBS.

Figure 3. Sephadex G-75 gel filtration chromatography of Zn-proteome from U87 mg cells exposed to apo-MT 2. (A) Control Zn-proteome, (B) Zn-proteome treated for 45 min with apo-MT 2. Reactions were run at room temperature under aerobic conditions.

Figure 4. Sephadex G-75 gel filtration chromatography of Zn-proteome from U87 mg cells exposed to EDTA. Reactions were run at room temperature under aerobic conditions.

Figure 5. Sephadex G-75 gel filtration chromatography of Zn-proteome from LLC-PK₁ cells induced to synthesize MT for 24 h with 80 μM Zn^{2+} and then exposed to TPEN. Conditions: plated cells (10^7 cells/plate) in 3 ml buffer exposed to 100 μM TPEN for 10 min at 37° C.

Figure 6.

Reaction of apo-MT with Zn-Sp1: EMSA Analysis. Conditions: A. human recombinant Zn₃-Sp1 + apo-MT (15 min) followed by addition of GC-1 for 15 min and then EMSA. B. Zn₃-Sp1 + GC-1 (15 min), then addition of apo-MT for 30 min and EMSA. C. Quantification of EMSA DNA bands.

Figure 7. Reaction of GSH with Zn-Sp1: EMSA Analysis. Conditions: A. human recombinant Zn₃-Sp1 + GSH (15 min) followed by addition of GC-1 for 15 min and then EMSA. B. Zn₃-Sp1 + GC-1 (15 min), then addition of GSH for 30 min and EMSA. C. Quantification of EMSA DNA bands.

Figure 8.

Reaction of TPEN with Zn-Sp1: EMSA Analysis. Conditions: A. human recombinant Zn₃-Sp1 + TPEN (15 min) followed by addition of GC-1 for 15 min and then EMSA. B. Zn₃-Sp1 + GC-1 (15 min), then addition of TPEN for 30 min and EMSA. C. Quantification of EMSA bands.

Figure 9.

EMSA for Zn-Sp1 in LLC-PK₁ cells treated with TPEN. Conditions: 30 μ M TPEN and GC-1 DNA Probe for Sp1. EMSA of control and treated cells includes antibody to Sp1.

Table 1Calculation of Apo-MT in U87 mg Cells^a

	MT Zn (nmol)	MT Cd (nmol)	Total Metal (nmol)	Apo-MT ^b (nmol protein)
<u>In vitro</u>				
Control	11		11	
Reaction	9	7	16	0.7
<u>In vivo</u>				
Control	12		12	
Reaction	12	6	18	0.9

^aConcentration per 10⁸ cells.^bApo-MT = [(MT Zn_{reaction} + MT Cd_{reaction}) - MT Zn_{control}][1/7]

Table 2
Reaction of Zn-Proteome with Apo-MT 2, GSH, and Synthetic Chelating Agents^a

Chelating Agent	Agent Conc. (μM)	Zn-Proteome Conc. (μM)	Log Conditional Stability Constant	Time (min.)	Zn-Proteome Reacted (%)
TPEN	100	50	15.6 [27]	30	38
EDTA	143	70		30	22
Apo-MT	160	120	13.4 [28]	30	32
	46 ^b	23	11.3 [7.8]	30	6
	30 ^b	15		45	3
	20 ^{b,c}	10		30	13
	100 ^{b,c}	50		30	0
EGTA	100	50	8.8[29]	30	34
GSH	2 mM	50	—	30	11
	2 mM ^c	70	—	30	9

^a Conditions of reactions: Room temperature, aerobic

^b Zinc binding sites

^c Conditions of reactions: Room temperature, anaerobic