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Reactivity of Zn-, Cd-, and Apo-Metallothionein with Nitric Oxide Compounds: In Vitro and Cellular Comparison

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Introduction

Mammalian metallothionein (MT) is a small, thiol rich protein that binds multiple metal ions in two metal-thiolate clusters [1]¹. Numerous studies, including ones with MT knock-out mice, demonstrate that the presence of MT in cells protects them from excessive exposure to metal ions such as Cd²⁺ [2,3]. Undoubtedly, this property is due to the capacity of MT with its many cysteinyl sulfhydryl ligands to bind various metal ions with high affinity and, thereby, to prevent them from interacting with sensitive sites in the cell [4]. It is also likely that the high density of MT=s thiolate groups reacts with and protects cells from a variety of reactive, electrophilic molecules, including oxidants such as hydrogen peroxide and hydroxyl radical [3,5].

According to NMR and X-ray crystallographic studies, Cd₇-MT and Cd_nZn_(7-n)-MT are comprised of two domains, each organized about a metal-thiolate cluster with stoichiometries of M(Cd/Zn)₄S₁₁ (C-terminal α-domain) and M(Cd/Zn)₃S₉ (N-terminal β-domain) [1,6,7]. The cluster structures are built upon the tetrahedral binding of the metal ions to cysteinyl thiolates and the presence of multiple sulfhydryl groups that bridge between metal ions and knit the clusters together. As the peptide wraps about each cluster, the overall solvent accessibility of

¹Abbreviations: apo-MT, metal ion free or metal ion unsaturated metallothionein; Cd- MT, cadmium metallothionein without reference to the number of bound Cd ions DEA/NO, 2-(N,N-diethylamino)-diazene 2-oxide; DETA/NO, 1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate; DTNB, 5,5'-dithiobis(2-nitrobenzoate); GSH, glutathione; DTT, dithiothreitol; GS-NO, S-nitroso-glutathione; LMW, low molecular weight (defined in **Figure 9**); PAR: 4-(2-pyridylazo)resorcinol; proteome, high molecular weight band of protein defined by Sephadex G-75 chromatography (defined in **Figure 9**); PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; RS-NO, general nitrosyl thiolate; MT, metallothionein; Zn-MT, metallothionein with focus on sites that have bound Zn²⁺; SH, thiol or sulfhydryl group; MTT, thiazolyl blue tetrazolium bromide; SNAP, S-nitrosoacetylpenicillamine; SNOC, S-nitrosocysteine; Zn₇-MT, fully Zn²⁺ saturated metallothionein; zincon, 2-carboxy-2-hydroxy-5-sulfoformazyl-benzene sodium salt

the thiolate sulfurs is substantially reduced [8]. Still, significant solvent exposure remains that provides the opportunity for reactions between MT and metal ions or electrophiles [8].

Several cellular investigations have suggested that MT is a locus of reaction for NO or its metabolites. Overexpression of MT in NIH 3T3 cells protects them from the toxicity of S-nitrosylacetylpenicillamine (SNAP) and nitric oxide synthase-generated NO [9]. A MT chimera with two different green fluorescent protein variants fused to its amino and carboxyl termini has been constructed such that the two fluorescent centers undergo fluorescent resonant energy transfer only in the holoprotein [10,11]. Transfection and expression of the gene for this chimeric structure has been carried out in sheep pulmonary artery endothelial cells and fibroblasts from MT normal and knock-out mice. Treatment of these cell types with S-nitrosocysteine reduced the efficiency of MT-based fluorescent resonant energy transfer, indicative of a reduction in the intracellular concentration of the native protein through reaction with NO species [10]. Exposure of Chinese hamster ovary cells, preinduced by Cd²⁺ to make MT, to DEA/NO resulted in the loss of 30–40% of MT cadmium binding sites [12]. A related experiment also showed that the NO agent, DETA/NO, released Zn²⁺ from cellular Zn-MT [13].

Only a few studies have inquired about the chemistry of reaction of MT with NO and S-nitroso-compounds. In the first report, the aerobic reaction of commercial Cd_nZn_(7-n)-MT with high concentrations of NO gas or S-nitrosocysteine, which rapidly decays to yield NO, liberated metal ions and modified sulfhydryl groups by S-nitrosation [14]. Under rigorously anaerobic conditions, Zn-MT also reacted with NO gas as measured by the appearance of free Zn²⁺ [15,16]. Although the formation of S-nitroso-groups could not be detected in the absence of O₂, the characteristic absorbance of this species was immediately detected once the system was opened to air [15]. In another experiment, using Cd₇-MT in the presence of DEA/NO, 30–40% of the Cd was released [12]. A related NMR study confirmed that DEA/NO reacted exclusively with the β-domain of Cd₇-MT [17]. Finally, it has been observed that one of the less common metallothionein isoform, MT-III, reacts more rapidly with S-nitrosocysteine than MT-I or -II. It was concluded that a transnitrosation reaction accounted for the reaction [18].

As a group, the in vitro results are not systematic or extensive. Thus, it is difficult to know how to interpret the reported cellular investigations focused on the reactivity of MT with NO species. In this context, the present study addresses the reactivity of various S-nitrosothiolates and NO and its oxidation products with Zn₇- and Cd₇-MT. We also considered the possible products of these reactions and the comparative reactivity of Zn₇-MT and apoMT with these NO compounds. To link these results with the cellular setting, experiments have also analyzed the impact of NO on the sulfhydryl and Zn²⁺ content of the proteome, MT and low molecular weight/ glutathione pools in two cell lines, TE-671, derived from a mouse medulloblastoma tumor, and LLC-PK₁, tubular cells from pig kidney.

Materials and Methods

Materials

Rabbit liver Zn₇-MT 2 (isoform 2) was isolated and purified as previously reported [19]. The purification of the protein was characterized by atomic absorption spectrophotometry and Ellman=s reaction with DTNB to determine the thiol content of the protein [20]. The Zn²⁺ to SH ratio was typically 2.9. The metal-free form of MT, apo-MT, was prepared by acidification of Zn₇-MT followed by Sephadex G-15 gel-filtration at pH 2. No zinc was detected in the product by atomic absorption spectrophotometry; the concentration of apo- MT was determined by thiol group analysis with DTNB. Samples of apo-MT were freshly prepared for each experiment and were kept at pH 2 until the experiment began. Cd₇-MT was prepared by reconstituting apo-MT with Cd²⁺ at pH 7.4 [21]. SNAP and PTIO were obtained from Sigma

(St. Louis, MO, USA); GSNO, DEA/NO were from Biotium (Hayward, CA, USA); zincon was from Aldrich (Milwaukee, WI, USA). Micro Bio-Spin 6 chromatography columns with an exclusion limit as 6,000 Daltons were purchased from Bio-Rad. Spectrophotometric measurements were carried out with a Beckman Coulter DU 640 UV-vis spectrophotometer.

Zinc Release Assay

Zn₇-MT and NO related compounds were mixed and incubated in cuvettes. After a given period of time, zincon, which does not react with native Zn₇-MT, was added as a chromophoric indicator of free zinc [22]. Zinc released from MT up to that time point was determined by measuring the absorbance of zinc-zincon complex at 620nm ($\epsilon_{620}=23,200 \text{ M}^{-1} \text{ cm}^{-1}$) [23]. The kinetics of the reaction were obtained by plotting the instantaneous Zn-zincon absorbance obtained at different time points during the course of reaction. This discontinuous method of kinetic analysis was used to measure free Zn²⁺ because the Zn-zincon complex gradually lost absorbance in the presence of various NO-compounds (data not shown). The typical condition for the zinc release assay was: 5–6 μM Zn₇-MT, 300–600 μM NO-donor compounds, 100 or 200 μM zincon, and 50 mM Tris-Cl buffer solution at pH 7.4 and 25° C.

Reaction of Apo-MT with NO-donors

Apo-MT was reacted with NO-donor compounds and the extent of this reaction ascertained by measuring the loss of zinc-binding ability of MT thiols in a modified zinc release assay. Apo-MT at pH 2 was brought to pH 7.4 by the addition of 2 M Tris base. Under anaerobic conditions, NO-donor compounds were then added and allowed to react for a given period of time. A known excess of zinc solution was introduced into the reaction mixture and the zincon indicator added. The absorbance of the Zn-zincon complex at 620 nm was immediately measured. It was assumed that only unreacted apo-MT retained zinc-binding ability in the presence of zincon. Thus, the extent of this reaction, assessed by the decreasing ability of Zn²⁺ to bind to MT, was the difference between the free Zn²⁺ at time t and that at time zero.

Light Effect upon the Reactions of MT with NO-donor Compounds

The role of light in the reactions of Zn₇- or Cd₇-MT with NO donor compounds was studied by conducting parallel sets of experiments in the dark and in the presence of light provided by two Philips light tubes (TYPE F32T8/TL735) which were positioned one meter above the reaction cuvettes. Zinc release was assayed as described above.

The Effect of PTIO in the Reaction of Zn₇-MT with DEA/NO: the Use of Spin Columns to Separate Metal Ions from Metallothionein

Under anaerobic conditions, 6 μM Zn₇-MT was reacted with 300 μM DEA/NO for 15, 30 and 80 minutes, respectively, in the presence or absence of 300 μM PTIO. The reaction mixture was then immediately passed through a Micro Bio-Spin chromatography column (Bio-Rad), following the user's instructions. The extent of reaction was determined by inductively coupled plasma-mass spectrometry (Micromass, Platform model) by measuring the Zn²⁺ content of the solution that passed through the membrane during centrifugation. As a control, > 90% of 6 μM Zn₇-MT was shown to be recovered from the spin column.

Cell culture

TE671 medulloblastoma cells were cultured on plates in Dulbecco's Minimal Essential Media (low glucose) supplemented with 75 U/L Penicillin and 50 mg/L Streptomycin at 37 ° C in a 5% CO₂ environment as previously described [24]. Bovine fetal serum concentration was 3.3% for maintenance cell culture and 5% for experiments. LLC-PK₁ cells were grown analogously in M199 media supplemented with Penicillin and Streptomycin. In this case the bovine fetal serum concentration was 4% during routine cell culture and 5% for the experiments. Both cell

types were exposed to a range of concentrations of Zn^{2+} for 24 h and were then checked for loss in viability. At concentrations up to $80 \mu\text{M}$ Zn^{2+} , there was no discernable decline in cell viability according to the MTT assay.

MTT Assay of Cell Viability

Cells were grown in 24 well culture plates in 1 mL of media. The MTT assay measures mitochondrial activity and the assumption is that only active, viable cells will be able to convert the yellow MTT to an insoluble blue metabolite that is solubilized and then measured spectrophotometrically [25]. A $100 \mu\text{L}$ aliquot of 12 mM MTT in phosphate buffered saline was added to the 1 mL of media in each well and mixed with gentle agitation. The plate was returned to the incubator for 2 h. Then, the medium was removed and 1 mL of acidified (0.04 N HCl) isopropanol was added to each well. The alcohol solution was agitated until all of the blue dye dissolved. The sample absorbance was measured at 570 and 630 nm with a UV-vis spectrophotometer and the difference (570 nm – 630 nm) calculated. The resulting data for DEA/NO treated cells were compared with untreated control cells.

Chromatography of TE671 and LLC-PK₁ Cells

Cells were rinsed, harvested, and homogenized with an ultrasonicator. The homogenate was then centrifuged at $50,000 \text{ g} \times 20 \text{ min}$ and the supernatant analyzed by gel filtration chromatography using Sephadex G-75 in degassed 20 mM Tris-Cl buffer [24]. Generally, this type of chromatography segregates thiols and metal ions into three aggregate pools, proteome, MT, and LMW. The individual eluate fractions were analyzed for sulfhydryl concentration using Ellman's assay (see above) and for Zn^{2+} or Cd^{2+} by atomic absorption spectroscopy. The total thiol and Zn^{2+} concentrations of the three pools were obtained by peak integration and normalized to 10^8 cells. The figures and tables show representative profiles of reactive sulfhydryl groups and Zn^{2+} .

Total and Metal Ion Free-metallothionein Determination

A number of cultured cells and animal tissues contain apo- as well as holo-metallothionein [26–28]. The Cd^{2+} saturation assay was used to determine the total metal binding capacity of metallothionein. It makes use of the very large affinity of Cd^{2+} for MT to saturate the protein with metal ion [26]. The assay was carried out by incubating half of the cell supernatant with Cd^{2+} and then comparing the total metal ion content of the MT fraction of control and Cd^{2+} treated samples after Sephadex G-75 chromatography. The Zn^{2+} content of the MT band dropped due to the Cd^{2+} displacement of the MT bound Zn^{2+} and metal-free SH groups associated with Cd^{2+} . Then the $\text{SH}:(\text{Zn}^{2+} + \text{Cd}^{2+})$ content of the peak was calculated using the value in parenthesis for $\text{Zn}^{2+} + \text{Cd}^{2+}$ listed in Table 2. Total MT was taken to be $(\text{Zn}^{2+} + \text{Cd}^{2+})/7$; total SH was $(\text{Zn}^{2+} + \text{Cd}^{2+}) \times 2.9$. Metal ion free SH groups were calculated as the total concentration of SH minus the zinc ion concentration times 2.9, the ideal ratio of SH to Zn^{2+} in $\text{Zn}_7\text{-MT}$.

Data analysis—All chemical experiments were carried out at least 3 times. Where fixed time measurements of reaction progress were made, averages $\pm$ standard deviations were calculated. Kinetic plots that illustrated qualitative rates of reaction were representative of these experiments. Because the results were clear cut in terms of the role of reaction variables, detailed statistical analysis was not applied. The cellular SH and Zn concentrations obtained by column chromatography and subsequent metal and sulfhydryl group analysis are representative of results with two cell types and several cultures of each. Replicate columns were run in order to obtain averages and ranges that illustrated the reproducibility of individual experiments with a given cell preparation.

Results

Introduction

The aim of the following chemical studies was to assess the reactivity of Zn₇-MT with S-nitroso-compounds as well as NO and its oxidation products (Table 1). With SNAP and GSNO, the transnitrosation reactivity of Zn₇-MT was assessed (reaction 1).

SNAP and GSNO are comparatively stable molecules. Under pH neutral conditions, they decompose in the dark, releasing NO with respective half-lives of hours and days [23]:

As a result in our studies, transnitrosation could be measured without complication from reactions involving NO and its oxidation products. By including light and oxygenation state as variables, further definition could be made about the nature of the reactive species in these reactions. Using DEA-NO as a source of NO with a decomposition half time of about 20 min, we directly tested the reactivity of MT sulphhydryl groups with NO (anaerobic conditions) or NO, NO₂ and N₂O₃ (aerobic conditions).

For these studies we employed a single MT isoform, MT-2 from rabbit liver, as representative of the commonly found MT-1 and 2 structures. These MT variants different only in the location of non-cysteine residues and have repeatedly been shown to display similar patterns of chemical reactivity. In order to test the relevance of the chemical results on cells, we chose two cell lines that are commonly used in our laboratory for studies involving metallothionein, TE671 human glioblastoma and LLC-PK₁ pig kidney proximal tubule cells.

Reaction of Zn₇-MT with SNAP-Requirement for Oxygen

The reaction of 6 μM Zn₇-MT with 300 μM SNAP was studied as a function of the presence of light and oxygen. Zn²⁺ release was detected by an increase in absorbance of the Zn-zincon complex at 620 nm (Fig. 1). In aerobic reaction mixtures exposed to light, more than 20% of the metallothionein-bound Zn²⁺ became available for zincon chelation (~8 μM Zn²⁺) within 60 minutes; nearly 40% was released within two hours. In parallel, the decomposition of SNAP, measured by the loss of absorbance of the RS-NO bond (λ_{max}, 338 nm), was shown to occur with kinetics similar to those for Zn²⁺ release (data not shown). In contrast, there was no observable formation of Zn-zincon under anaerobic conditions in the presence of light, indicating that Zn₇-MT did not undergo a reaction with NO, the decomposition product of SNAP, in the absence of O₂ (Fig. 1). Nevertheless, light-exposed SNAP, itself, was degraded in the absence or presence of oxygen. The same O₂ requirement for the effective liberation of Zn²⁺ from Zn₇-MT was defined in the reactions of Zn₇-MT with GSNO and DEA/NO as described below. This indicated that the O₂ requirement is a general property of these reactions of Zn₇-MT with NO-donor compounds.

Light as a factor in the reaction of Zn₇-MT with SNAP

Light sensitivity is a common property for many NO-donor compounds including SNAP [29, 30]. Using the same reaction conditions as in Fig. 1, very little zinc release was observed when light was excluded from the aerobic reaction mixture (Fig. 2). During the first two hours, only about 3% of the Zn²⁺ was released; after 6 h approximately 10% had been mobilized. Similarly, in the absence of light, there is little observable decomposition of SNAP. There was no change in absorbance at 338 nm during the entire reaction period. An hypothesis consistent with these

results is that an S-nitroso-thiolate interchange reaction occurred as follows, which displaced Zn^{2+} from MT without altering the total concentration of RS-NO:

With the introduction of light into the system, the reaction was activated and nearly 40% of MT-bound zinc was released and detected as the Zn-zincon complex in the first two hours as in Fig. 1.

Reaction of GSNO with Zn₇-MT

GS-NO has been proposed as the major storage form of bioactive NO in the cell [29,30]. The reported half-life of GS-NO varies widely, ranging from minutes to days [23,29]. It has been suggested that the cause of this discrepancy arises from the differential contamination of samples of GS-NO with trace amounts of metal ions, particularly Cu^+ ion, during its preparation or handling. In our experiments, the half-life of GS-NO at pH 7.4 was very long, on the order of days, under aerobic conditions in the dark.

The characteristics of the reaction of Zn₇-MT with GS-NO were similar to those with SNAP except the reactions occurred more slowly. Thus, after 2 h of aerobic reaction of 6 μM of Zn₇-MT and 300 μM of GS-NO in the presence of light, only 5% of the total Zn^{2+} was released. Extending the reaction time to 5 hours increased the extent to 13%. When the concentration of GS-NO was doubled to 600 μM Zn^{2+} release increased to 8% in 2 hours and 18% in 5 hours. No Zn^{2+} was released in the absence of O_2 (data not shown) or light.

Reaction of DEA/NO with Zn₇-MT

DEA/NO is a widely used NO-donor compound, which spontaneously and rapidly releases NO under physiological conditions in the absence of light or oxygen [29]. In the present study, the half-life for DEA/NO was about 20 minutes at pH 7.4 and 25 °C. As in other reactions, 6 μM of Zn₇-MT was allowed to react with 300 μM DEA/NO (Fig. 3). Considering the degradation kinetics of DEA/NO, about 540 μM NO can be generated within 1 h. The same O_2 dependence of Zn^{2+} release from MT was observed for the DEA/NO reaction as was observed with the other, less reactive NO compounds. Thus, under aerobic conditions, 18% of the total Zn^{2+} was detected by zincon after 1 h; however, under anaerobic conditions, no zincon chelatable Zn^{2+} could be measured after 4 h (Fig. 3). Therefore, in this experiment, it was particularly clear-cut that NO, itself, did not react with zinc-metallothionein.

The possible influence of light on the reaction was also investigated. In contrast to the reactions involving SNAP and GS-NO (Fig. 1), the rate of reaction was not dependent on a photochemical event during the 1 hour reaction time. In the presence or absence of light, the extent of Zn^{2+} mobilization was the same, provided that the same aerobic experimental conditions were used. Unlike SNAP and GS-NO, which remained reactive for hours, extending the reaction time of Zn₇-MT with DEA/NO achieved only modest gains in Zn^{2+} release (9% over the next 3 h).

Effect of PTIO in the reaction of Zn₇-MT with DEA/NO

Experiments were conducted to probe the nature of the reactive species in the aerobic reaction of DEA/NO with Zn₇-MT. It was hypothesized that the reaction of NO with O_2 generated NO_2 and that this species might be reactive with the protein (reaction 2). Particularly, under conditions of limiting NO, subsequent conversion to N_2O_3 would be hindered (reaction 3)

In order to assay the reactivity of NO_2 with Zn-MT, PTIO was added to the reaction mixture. It reacts readily with NO according to reaction 4 [31]:

Spin-column chromatography was used to evaluate the labilization of Zn^{2+} from Zn₇-MT during its reaction with DEA/NO in the absence and presence of PTIO (Fig. 4). As anticipated from the results obtained with zincon, an insignificant amount of Zn^{2+} was released under anaerobic conditions in the absence of PTIO. There was also no evidence of direct reaction between Zn₇-MT and PTIO. In contrast, when Zn₇-MT was allowed to react with a mixture of DEA/NO and PTIO under anaerobic conditions, significant Zn^{2+} release was observed even at the first time point and about 75% of complete reaction occurred within 80 min. Therefore, PTIO effectively substituted for O_2 , leading to the generation of NO_2 , which showed significant reactivity with the Zn-sulfur clusters in the Zn₇-MT. In this experiment, 300 μM PTIO actually functioned much better than O_2 in air ($\sim 250 \mu\text{M}$) as an oxidizing agent for NO.

The product of the anaerobic reaction of Zn₇-MT with DEA/NO and PTIO was reacted with DTT in order to see whether this dithiol reagent could reverse the initial reaction. According to Fig. 5, exposure to DTT completely restored the Zn^{2+} binding capacity of the MT product. In comparison, the product without DTT treatment displayed progressively reduced affinity for Zn^{2+} . Obvious hypotheses are that DEA/NO plus PTIO oxidized the sulfhydryl groups of MT and DTT effected their reduction or that S-nitrosothiolates had formed and an S-nitrosyl transfer from MT to DTT had occurred.

Rates of Reaction of Zn₇-MT with SNAP in the Presence and Absence of GSH

The reaction of Zn₇-MT with SNAP was conducted in the presence and absence of GSH in order to understand the comparative reactivity of these two prominent thiol compounds that may compete in cells for electrophilic NO compounds. The presence of 200 μM GSH inhibited the light dependent reaction of Zn₇-MT with SNAP (Fig. 6). Similar inhibition of Zn^{2+} release by GSH in the reaction of Zn₇-MT with DEA/NO was also observed (data not shown).

Reaction of Cd₇-MT with SNAP and DEA/NO

The reactions of Zn₇-MT and Cd₇-MT with SNAP are compared in Fig. 7. Although the rates of each reaction were similar in the presence of oxygen and light, the extent of reaction of the Zn-protein was much greater. It reached 80% based upon the production of zincon chelatable Zn^{2+} , whereas, only 33% of the Cd^{2+} was released as measured with the chromophoric dye, PAR [22]. A similar incomplete reaction was observed between DEA/NO and Cd₇-MT; repeated additions of 300 μM DEA/NO could not push the reaction further. The extent and limit of the Cd₇-MT reaction with DEA/NO was consistent with only the Cd₃- β domain undergoing reaction. In contrast, both domain clusters of Zn₇-MT underwent reaction.

Reaction of Apo-MT with SNAP and DEA/NO

It has commonly been assumed that there is little or no apo-MT in the cell. However, significant amounts of apo-MT have been discovered in numerous tumor cell lines [26]. Recently, this finding was extended to normal, mammalian tissues and cell lines over-producing MT [27, 28]. Therefore, we tested the reactivity of apo-MT with SNAP and DEA/NO. Apo-MT was able to react with both kinds of NO-donors in the dark and in the absence of oxygen; the reaction

with SNAP was 80% complete in about 5 min (Fig. 8). Unlike the comparable reactions of Zn²⁺-MT, there was no O₂ or light dependence to the reactions with apo-MT. This experiment showed that apo-MT was much more reactive than the Zn-saturated protein and that different reaction pathways were followed when it reacted with these NO-donor compounds.

These in vitro results implied predictions of in vivo reactivity of Zn- and apo-MT sites. The following experiments examined the sulfhydryl reactivity of MT in comparison with other major pools of thiol groups with DEA/NO in two cell lines under control and Zn²⁺ induced conditions that elevated the concentration of metallothionein.

Effect of DEA/NO on TE671 and on LLC-PK₁ Cell Viability

TE671 cells exposed to DEA/NO for 24 h did not display reduced viability up to 0.45 mM according to the MTT assay. Above that concentration, viability gradually decreased: a 15% decline was observed with 1 mM DEA/NO and more than 80% of the cells lost viability when the DEA/NO concentration reached 10 mM. Cell viability also began to drop in LLC-PK₁ cells as the DEA/NO concentration was increased above 0.45 mM. Preincubation with Zn²⁺ to induce MT did not alter the viability of either cell line at 0.45 mM DEA/NO.

Thiol and Metal Ion Distribution in Control Cells

Cell supernatant from TE671 cells was chromatographed over Sephadex G-75 in order to quantify the thiol and Zn²⁺ associated with the proteome, metallothionein, and LMW/glutathione pools (**Figure 9a**). Both thiol and Zn²⁺ assays showed similar patterns with two distinct peaks. The first peak (proteome) in fractions 10–25 contained proteins with molecular weights generally greater than 30,000. The second peak enclosing fractions 35–45 represents the small molecules and includes GSH. MT, with a molecular weight of 6–7 kD, eluted at the position centered on fraction 30, exhibiting a small TNB absorbance band or shoulder between the other two peaks. Control LLC-PK₁ cells displayed a qualitatively similar profile, with distinct proteome and LMW bands of sulfhydryl groups and Zn²⁺ and little evidence of MT between them.

Peak integrations for the TE671 chromatographic peaks showed that per 10⁸ cells, 1230 and 480 nmol of thiols were associated with the proteome and LMW pools, respectively. In contrast, the putative MT cysteine content contributed only 42 nmol (Table 2). Recognizing that traces of SH and Zn²⁺ in the MT part of the chromatographic profile do not necessarily represent MT, Cd²⁺ saturation experiment was conducted to determine the total concentration of MT. Some, but perhaps not all, of the SH groups could be assigned to MT. In any case, the thiol concentration in the MT region was at best a minor component of the cytosolic complement of sulfhydryl groups in the TE671 control cells. Table 2 reveals a similar pattern of thiol and Zn²⁺ quantification among the components of LLC-PK₁ cell supernatant.

The presence of a sizable low molecular weight Zn²⁺ pool was notable. It precedes the thiol/glutathione sulfhydryl peak and thus does not represent free Zn²⁺ or a Zn-glutathione complex. Its identity is unknown but is under investigation.

MT Induction by Zn²⁺

MT content was clearly boosted in both sulfhydryl and Zn²⁺ when the TE671 cells were preincubated with 80 μM Zn²⁺ for 24 hours. Discrete MT peaks were revealed by both the DTNB and the Zn²⁺ analyses (**Figure 9b**). The thiol and Zn²⁺ contents of the proteome and LMW pools were unaffected by 24 h exposure to Zn²⁺. In contrast, the MT thiol content was boosted more than 5 fold, increasing from 42 nmol to 195 nmol. The Zn²⁺ content in MT band also increased from 4 to 41 nmol. The MT SH:Zn²⁺ of 4.8 indicated that much of the protein was not saturated with Zn²⁺. This was confirmed with the Cd²⁺ saturation experiment that

showed that the $\text{SH}:\text{Zn}^{2+} + \text{Cd}^{2+}$ ratio was 2.9, indicating that the total SH content of the band was assignable to MT.

In the case of LLC-PK₁ cells, incubation for 24 h with 80 μM Zn^{2+} did not impact the proteome or LMW thiol or Zn^{2+} concentrations. It did, however, greatly increase MT thiol content from 54 to 600 nmol/10⁸ cells. Although the corresponding Zn^{2+} concentration was elevated 13 times, the SH/metal ion ratio of the protein was 9.2, indicative that most of the protein was not bound to Zn^{2+} .

DEA/NO Reaction with Cell Thiols in the Absence and Presence of MT Induction

Incubation of DEA/NO with control TE671 cells caused a substantial loss of 330 nmol SH/10⁸ cells from the proteome pool when compared with untreated control cells (Table 2). The LMW band also lost 150 nmol free thiols during the DEA/NO treatment. The very small MT pool of sulfhydryl groups was relatively unaffected by the DEA/NO treatment in the thiol assay (Table 2). Indeed, when we conducted the Cd^{2+} saturation experiment to determine the total concentration of MT, it appeared that within the range of results, none of the pools displayed changes in Zn^{2+} content.

In the TE671 cells preincubated with 80 μM Zn^{2+} , the concentration of MT was elevated almost 5 fold. Based on the Zn^{2+} content, 76 and 119 nmol SH/10⁸ cells of free and metal ion bound sulfhydryl groups respectively comprised the MT peak. The DEA/NO treatment induced similar reductions in proteomic and LMW sulfhydryl groups as seen in the non-induced cell population. In addition, an increasing amount of MT thiols underwent reaction. More Zn^{2+} was labilized from the proteome; but little if any Zn^{2+} was lost from the MT band, though proteomic Zn^{2+} declined. Thus, in cells exposed to 80 μM Zn^{2+} , the sulfhydryl group content dropped from 195 to 160 nmol per 10⁸ cells. Since the Zn^{2+} content of the band did not change, these SH groups must be ones not ligated to Zn^{2+} . These results supported the hypothesis that DEA/NO reacted primarily with apo- or metal ion-free thiols in MT not with sulfhydryl groups associated with $\text{Zn}_7\text{-MT}$. That the sulfhydryl groups involved stemmed from MT protein was confirmed in the Cd^{2+} saturation experiment, which showed that $\text{SH}:(\text{Zn}^{2+} + \text{Cd}^{2+})$ ratio in the MT pool was 3.1 after DEA/NO treatment. This result was consistent with the all of the remaining SH groups being identified with MT. Notably, only about half (35 of 76) of the apo-SH groups underwent reaction with DEA/NO.

The impact of exposure to 0.45 mM DEA/NO for one hour on LLC-PK₁ cellular thiol content and distribution was examined by comparing results in control and DEA/NO treated cells in the absence and presence of metallothionein, induced by 80 μM Zn^{2+} . DEA/NO treatment of control LLC-PK₁ cells caused significant thiol loss from both the proteome and LMW bands. In the experiments shown, per 10⁸ cells, 240 nmol SH underwent reaction within the proteome as well as 70 nmol SH among the LMW species. In contrast, the small MT band showed a negligible change in the thiol measurement (Table 2). No significant change in Zn^{2+} was incurred in the MT fraction.

In the cells incubated with 80 μM Zn^{2+} , a large concentration of MT was induced. According to the $\text{SH}:\text{Zn}^{2+}$ ratio of 9.2 much of the band contained MT without bound Zn^{2+} . About 410 nmol of this thiol pool were not ligated to Zn^{2+} (Table 2). The DEA/NO treatment depressed proteomic and LMW sulfhydryl group concentration as in control cells not exposed to Zn^{2+} . It did so without altering the MT Zn^{2+} complement. Therefore, the major reduction in MT thiol content resulted from the reaction of DEA/NO with apoMT not $\text{Zn}_7\text{-MT}$. Nevertheless, its $\text{SH}:\text{Zn}^{2+}$ ratio remained above that for $\text{Zn}_7\text{-MT}$ at 4.2, indicating that metal-free sulfhydryl groups remained in the MT pool. This was confirmed with the Cd^{2+} -saturation assay, in which the resultant $\text{SH}:(\text{Zn}^{2+} + \text{Cd}^{2+})$ ratio declined to 3.4 as Cd^{2+} became bound to these metal-unsaturated sites.

Discussion

Nitric oxide is an important molecule involved in many physiological and pathological processes. Among them, covalent modification of sulfhydryl groups through S-nitrosation has been proposed as a major redox-based signaling process [32]. Metallothionein is a small protein with an extraordinarily large content and density of cysteine sulfhydryl groups [5]. Because of this peculiar property as well as the potential significance of MT in the metabolism of electrophilic agents and Zn^{2+} , the hypothesis that MT might participate in NO signaling and in the control of NO-based toxicity has been proposed and experimentally probed [3,9–14, 33–37].

These studies have concluded that metallothionein is reactive with NO or NO-related compounds. But there has been relatively little effort to further the understanding of the nature and extent of reactivity of metallothioneins with various NO donor species or to compare its reactivity with other intracellular thiol pools. Previous in vitro inquiry into the reactivity of MT with NO-related compounds utilized Cd_7 - and Zn_7 -MT in conjunction with NO gas or an S-nitroso-compound, SNOC, that rapidly releases NO [14–18]. In our experiments, we used GSNO, SNAP, and DEA/NO with half-times for NO release of days, hours, and minutes, respectively. With this range of structures, it was possible to assess the reactivity of Zn_7 - and Cd_7 -MT with S-nitroso-compounds as well as with NO, itself, under different conditions, including spontaneous and light-dependent decomposition (reactions 7 and 8).

These S-nitroso structures could be involved in different reactions with thiols, such as transnitrosation (reaction 9) and production of disulfide with liberation of $\text{NO}^{\cdot}$ (reaction 10):

Our results, summarized below, present a consistent picture, in which none of the NO donor compounds reacts directly with Zn_7 -MT at a substantial rate when measured anaerobically in the dark, such that light-dependent homolysis of the S-nitroso bond could not occur (reaction 8). Nor were their reactions accelerated when reacted with Zn_7 -MT in the light but in the absence of O_2 . Thus, the S-nitroso species, SNAP, and NO, itself, which was generated at varying rates with the three NO-donors, do not react significantly with Zn_7 -MT even when present in relatively high concentration and large excess with respect to the protein. Our finding of the aerobic reactivity of Zn_7 -MT with NO qualitatively agrees with Kroncke's original report [14]. However the lack of reactivity under anaerobic conditions appears to contradict reports indicating that Zn_7 -MT can react directly with NO [14,15].

Zn^{2+} was displaced from MT by GSNO or SNAP when both light and O_2 were present, conditions that both produce NO and support its oxidation (Fig. 1 and Fig. 2). Similarly, rapid decay of DEA/NO in the light or the dark but only in the presence of O_2 also destroyed the

MT Zn-thiolate clusters and released Zn^{2+} (Fig. 3). Hence, it was evident that further reaction of NO was necessary to produce a species that was reactive with $\text{Zn}_7\text{-MT}$.

The lack of much direct reactivity of SNAP or GS-NO with $\text{Zn}_7\text{-MT}$ differs from the results reported by Maret and coworkers, in which it was argued that the release of Zn^{2+} from MT isoforms I-III in their aerobic reactions with S-nitrosocysteine was caused by transnitrosation (reaction 7) [18]. It is possible that the three S-nitroso compounds differ substantially in their intrinsic reactivity with $\text{Zn}_7\text{-MT}$. Their conclusion was based on the finding that several chelating agents including zincon, the reagent used to measure liberated Zn^{2+} in their reactions as well as ours, stabilizes the S-nitroso bond against hypothetical adventitious Cu-dependent homolysis, thereby ruling out the participation of NO or its oxidation products. Complicating this interpretation was the observation that zincon, which binds Cu with low affinity in comparison with MT, was more effective in preventing S-nitroso bond cleavage than any isoform of metallothionein that has a very large affinity for Cu.

The similarity of the rates of degradation of $\text{Zn}_7\text{-MT}$ and of light dependent breakdown of SNAP or GS-NO S-nitroso bonds indicates that the rate limiting chemistry in these reactions is the cleavage of the S-nitroso bond (Fig. 2). This implies that the further reactions of NO with O_2 and the subsequent reaction of nitrogen oxide species with the metal-thiolate clusters are faster and non-rate limiting. S-nitroso-species may be produced as follows in reactions 4,5, and 11:

The modified MT might rapidly react intramolecularly and form disulfides with the displacement of NO^- as in reaction 12.

We tested this pathway using DEA/NO as a direct NO generator. As expected, oxygen was a necessary reactant with NO in order to degrade MT Zn-thiolate clusters (Fig. 3). Another experiment demonstrated that the substitution of an oxygen atom donor, PTIO, for O_2 in the DEA/NO reaction with $\text{Zn}_7\text{-MT}$ results in a relatively rapid degradation of the Zn-thiolate clusters (Fig. 4). Clearly, NO_2 that was produced in the reaction of NO with PTIO was an effective reactant with the protein. That the O_2 -dependent reaction is less efficient suggests that the products of the reaction of NO and O_2 are less reactive with $\text{Zn}_7\text{-MT}$ than NO_2 (reactions 9,10). By inference, N_2O_3 may be a significant product in the aerobic reaction. Considering that the aerobic reaction is sluggish in comparison with the anaerobic-PTIO reaction, N_2O_3 may not be the species that is primarily reactive with the clusters. Instead, we propose that Zn-MT reacts principally with NO_2 according to reaction 13:

The modified metallothionein that resulted from the reaction of NO₂ with Zn-MT could be wholly restored to its metal binding form by incubation with DTT (Fig. 5). The simplest explanation is that the initial reaction oxidized the sulfhydryl groups of MT to disulfides, leading to the dissociation of Zn²⁺ (reaction 14). Then, DTT reduced the oxidized protein through a thiol-disulfide interchange reaction:

The experiments can not rule out the possibility that DTT competed successfully for MT-bound S-nitroso groups. Nevertheless, since the reactive species appeared to be NO₂ not NO, the formation of MT-S-NO, did not seem likely.

The capacity of SNAP and DEA/NO to react with Zn₇- and Cd₇-MT was compared in order to follow up the earlier observations that DEA/NO only liberates Cd²⁺ from the Cd₃S₉ β-domain [12,17]. Our results qualitatively agree with this conclusion. The origin of this divergence in behavior of the two Cd-thiolate clusters is unclear. It does not appear to result from a thermodynamic discrimination between the Zn- and Cd-proteins. Reaction 11 may be thought of as a combination of metal ion dissociation to yield apo-MT followed by oxidation-reduction to produce disulfide and nitrite. Since the redox process for the apo-protein is identical for each reaction, a thermodynamic difference could only reside in the dissociation step. However, each metal-thiolate cluster of Cd₇-MT binds metal ions several orders of magnitude more strongly than those in Zn₇-MT [38]. Thus, the difference in behavior of the Zn₄S₁₁ and Cd₄S₁₁ clusters can not result from differences in metal-thiolate bond strength; otherwise the same lack of reactivity would be anticipated for the Cd₃S₉ cluster. The alternative is that a major kinetic barrier blocks the reaction of Cd₄S₁₁ but is absent in the case of Zn₄S₁₁. Although quantitative differences in reactivity of the α- and β-domains with reagents such as EDTA and DTNB have been noted based on the nature of the metal ion involved, the dramatic lack of reaction observed here and in previous experiments is unusual if not unique [19,39,40].

One way to assess the potential importance of metallothionein as a thiol-rich site of reaction of NO-compounds in cells is to compare its reactivity with that of the principal low molecular weight pool of sulfhydryl groups, GSH. This was done by examining the reaction of SNAP with Zn₇-MT in the presence and absence of GSH. Competing 80 or 200 μM GSH against 120 μM MT thiol, the extent of Zn²⁺ release was lowered 37% and 55%, respectively. Thus, GSH is an effective competitor for reactive NO-species such as NO₂ at similar concentrations of the two types of thiols. Considering that cellular glutathione concentrations are commonly in the mM range, it is difficult to argue that Zn₇-MT would be the sole, let alone preferred, site of reaction of NO₂.

The reactions of apo-MT with SNAP or DEA/NO were strikingly faster than those with Zn₇- or Cd₇-MT (Fig. 8). For example, the rate enhancement in the SNAP reaction that was realized by the substitution of apo-MT for Zn₇-MT was about 20 fold. Furthermore, like the reactions of GSH, those of apo-MT occurred in the dark and did not require O₂. Not surprisingly, NO and oxidized forms of NO were much more reactive with the free sulfhydryl groups of apo-MT than with the metal-bound thiols of the holoprotein.

It has been established that apo-MT is present in a number of tumor and normal cell types [26,27]. Recently, substantial pools of apo-MT have been discovered in cells induced to synthesize elevated concentrations of MT [28]. Together with the results on the comparative reactivity of apo- and Zn₇-MT, these observations support the hypothesis that NO compounds may target cellular apo-MT.

Overall, the reactivity of Zn₇-MT with SNAP and DEA/NO was not impressive in light of the hypothesis that it is a key site of reaction with such compounds in vivo. Thus, in order to make the connection between the observed chemistry and cellular responses to NO compounds, two cell lines and different intracellular MT concentrations were used to investigate MT as a potential site of reaction of DEA/NO.

The findings with the two cell types were qualitatively similar. Neither was very sensitive to DEA/NO under aerobic conditions; as a result, overt toxicity was not observed until its concentration increased above 0.45 mM. Nevertheless, even at 0.45 mM DEA/NO, sizable fractions of the cell's aggregate thiol content reacted with NO or its oxidation products. About 30% in TE671 cells and 15% in LLC-PK₁ cells (Table 2). Included among the reactive species were proteomic and low molecular weight thiols, the latter probably constituted primarily of glutathione. However, Zn₇-MT was not a significant target of DEA/NO at elevated concentrations of MT, including one involving LLC-PK₁ cells induced with 80 μM Zn²⁺, in which the MT pool represented about 25% of the sulfhydryl groups in the cell supernatant (Table 2). In contrast, metal-free, MT thiols did react with the NO species that were generated in situ in both TE671 and LLC-PK₁ cells (Table 2).

These results closely resemble the in vitro findings: MT sites with bound Zn²⁺ were unreactive with DEA/NO; metal-free MT-SH groups displayed significant reactivity. The comparative findings forge a strong link between the in vitro chemistry and the cellular behavior of MT in the presence of DEA/NO. In addition, they support the formation of NO₂ in cells exposed to NO.

In order to compare the reactivity of various classes of thiol groups, Figure 10 plots the concentration of reacted SH groups in each pool vs. its total concentration from Table 2. In the case of TE671 cells, the linear character of the graph indicate that thiol reactivity was similar for each type of thiol and that the extent of reaction was simply proportional to the sulfhydryl concentration of the pool. Notably, in the 80 μM Zn²⁺ treated cells, less than half of the MT unbound thiol groups underwent reaction (35 of 76). Despite the much enhanced in vitro reactivity of apoMT with DEA/NO, such sites were not selectively reactive in comparison with other thiol pools.

About 8% of the reactive SH groups were derived from apoMT in TE671 cells incubated with 80 μM Zn²⁺. This increased substantially to nearly 50% with LLC-PK₁ cells pretreated with 80 μM Zn²⁺. In this case, when apo-MT was present in large concentrations in comparison to other cellular thiols (ca. 25% of total), apo-MT was a selective site of reaction as shown by its position above the line. Although there was preferential reaction of DEA/NO with apo-MT, metal ion-unsaturated MT never approached becoming the sole site of reaction of DEA/NO.

In neither cell type did the MT thiol pool substantially spare the proteomic sulfhydryl group from reaction. The results also indicated that the LMW sulfhydryl fraction, which contains glutathione, did not preferentially react with DEA/NO. Thus, neither of the putative protective thiol pools in cells, MT and GSH, totally ablated reaction with proteomic sulfhydryl groups even when excesses of each were present in the cell in comparison with the total concentration of thiol groups that underwent reaction.

The degree to which thiols in the functional protein pool were altered by DEA/NO was surprising, with 18–27% being modified in the control cells, particularly because there was no apparent effect of DEA/NO on the short term 24 h viability of either cell line. The combination of the widespread modification of cellular SH groups by DEA/NO and the NO donor's lack of toxicity demonstrated that at least over a 24 h period these cells were remarkably unimpaired by the initial modification of a substantial fraction of their protein sulfhydryl groups. The result

suggests that the proteome reservoir of sulfhydryl groups must be considered along side MT and GSH pools as a major site of reaction with NO related compounds.

These findings raise the question of the mechanism of selectivity that numerous studies describe when NO species react with specific proteins in the cell [41]. Based on the present results, specificity is only apparent and reflects a focus on NO-based reactions with particular proteins without reference to the much wider reactivity observed within the aggregate proteome. This conclusion leaves unresolved and, indeed, emphasizes the question how selective NO signaling occurs within the broadly reactive proteome pool of SH groups. The widespread reactivity of NO with sulfhydryl groups parallels the recent findings by Liebler et al on the widespread reactivity of other electrophiles with proteomic sulfhydryl groups [42].

Analysis of the effects of DEA/NO on the two cell populations included a determination of its impact on the Zn^{2+} content of each pool. NO or products of its reaction with oxygen did not alter the concentration of Zn^{2+} in the induced MT pool (Table 2). Nor did it affect LMW Zn^{2+} . In both cell types, a consistent trend was observed in which DEA/NO lowered Zn^{2+} in the proteome fraction. In sum, the present results failed to show that MT is a sensitive source of labile Zn^{2+} in the presence of an NO donor. Nevertheless, previous reports single out MT as the site that liberates metal ions upon reaction with NO compounds [10,11,43]. Considerably more work will need to be done to reconcile the direct measurements of MT reactivity with NO described in this study with other reports of the involvement of metallothionein in NO trafficking and metabolism.

In summary, cellular results agree with in vitro observations of the sluggish reactivity of Zn_7 -MT with NO and its oxidative products. They also show that apoMT is reactive with aerobic DEA/NO. Nevertheless, these findings do not support a unique role for Zn_7 -MT or even its metal unsaturated species in NO metabolism. The results also reveal that widespread modification of cellular thiols by NO species can occur without acute negative effects on cell viability.

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Figure 1. Oxygen requirement of reaction of Zn₇-MT with SNAP

Conditions: 6 μ M Zn₇-MT, 300 μ M SNAP, plus 200 μ M zincon in 50mM Tris-Cl buffer at pH 7.4 and 25 °C. The maximum possible absorbance is approximately 1.0. (▲, aerobic; ■, anaerobic)

Figure 2. Light dependence of SNAP decomposition and Zn²⁺ release

Conditions: 6 μM Zn₇-MT, 300 μM of SNAP, plus 200 μM zincon in 50mM Tris-Cl buffer at pH 7.4 and 25 °C under aerobic conditions. The absorbance decrease at 338 nm due to the loss of the S-nitroso bond of SNAP in the reaction mixture. (■, dark; ▲, light) and the Zn²⁺ release, measured by the formation of the Zn-zincon chromophore at 620 nm. (□, dark; △, light)

Figure 3. Reactions of DEA/NO with Zn₇-MT

Conditions: 6 μ M Zn₇-MT, 300 μ M DEA/NO plus 200 μ M zincon in 50mM Tris-Cl buffer at pH 7.4 and 25 °C for 1 hour. Reactions were conducted in either light or dark. Effect of O₂ on the release of free zinc from MT. Comparison of the Zn-zincon absorbance under aerobic and anaerobic conditions. (■: aerobic; □: anaerobic)

Figure 4. The effect of PTIO in the reaction of DEA/NO with Zn₇-MT

Conditions: 6 μ M Zn₇-MT, 300 μ M DEA/NO, 300 μ M PTIO in 50 mM Tris-Cl buffer, at pH 7.4 and 25 °C under aerobic conditions. At intervals, the reactions of Zn₇MT with $\blacktriangle$, DEA/NO; $\blacksquare$, PTIO; and $\blacklozenge$, DEA/NO+PTIO were separated with spin column chromatography and the separated free Zn²⁺ concentration measured by ICP-MS after 60 fold dilution..

Figure 5. Properties of the product of NO and PTIO with Zn₇-MT: the effect of DTT

Conditions: 6 μ M Zn₇-MT, 300 μ M DEA/NO, 300 μ M PTIO in 50 mM Tris-Cl buffer, at pH 7.4 and 25 °C under anaerobic conditions. After 30 and 60 min, 0.5 mM DTT was added to the mixture. After a 5 minute incubation the mixture was separated with spin column and Zn measured by ICP-MS. (■, with DTT reduction; ◆, without DTT reduction)

Figure 6. Effect of GSH on the reaction of SNAP with Zn₇-MT under aerobic conditions
Conditions: 6 μM Zn₇-MT, 300 μM of SNAP, in 50 mM Tris-Cl buffer at pH 7.4 at 25 °C in the presence or absence of 80 or 200 μM GSH for one hour. Zn²⁺ release measured by the formation of the Zn-zincon chromophore at 620 nm. (■, dark; □, light)

Figure 7. The reactions of SNAP with Zn₇-MT and Cd₇-MT

Conditions: 300 μ M SNAP, 6 μ M Zn₇-MT or 6 μ M Cd₇-MT in 50 mM Tris-Cl buffer, at pH 7.4 and 25 °C under aerobic conditions. The release of metal from MT was followed spectrophotometrically with 200 μ M zincon (Zn) at 620 nm or 200 μ M PAR (Cd) at 486 nm (ϵ_{486} of Cd-PAR = 4.2×10^4 M⁻¹ cm⁻¹). (■, Zn₇-MT; ▲, Cd₇-MT)

Figure 8. Reactions of apo-MT with NO compounds

Conditions: 4 μM apo-MT, 300 μM SNAP or DEA/NO, in 50 mM Tris-Cl buffer at pH 7.4 and 25 $^{\circ}\text{C}$ under anaerobic conditions. Free Zn^{2+} in solution was measured with 200 μM zincon after the addition of 40 μM Zn^{2+} to the reaction mixture to bind to the remaining apo-MT. For comparison, the same procedure was applied to 6 μM $\text{Zn}_7\text{-MT}$ with 300 μM SNAP. The data is presented as the % of the possible absorbance at 620 nm. (■, Zn_7MT with SNAP; ▲, Apo-MT with SNAP; ●, Apo-MT with DEA/NO, 30 min point only)

Figure 9. Sephadex G-75 gel filtration column chromatography separation of TE671 cell supernatant
Control cells (a) and cells incubated with 80 μ M ZnCl₂ for 24 h (b) were chromatographed and the fractions tested for zinc (■) using atomic absorption spectrophotometry and SH (◆) using the DTNB colorimetric assay which measures the absorbance of the reagent at 412 nm. The three major zinc peaks, Zn-proteome, MT and LMW are indicated.

Figure 10. Cell thiol pools: relationship of SH reactivity to concentration

Data for TE671 (filled symbols) and LL-CPK₁ cells (open symbols) taken from Table 2. (■, □, proteome SH; ●, ○, SH; ▲, △, LMW (glutathione) SH)

Table 1

NO Reagents and Their Properties

Compound	NO Release, $t_{1/2}$
$\begin{array}{c} \text{O}^- \\ \\ \text{ON}-\text{N}-\text{N} \\ \quad \diagup \\ \text{CH}_2\text{CH}_3 \\ \\ \text{CH}_2\text{CH}_3 \end{array}$ <p>DEA/NO</p>	20 minutes, This work
$\begin{array}{c} \text{COO}^- \\ \\ \text{ON}-\text{SC}(\text{CH}_3)_2\text{CH} \\ \\ \text{NHCOCH}_3 \end{array}$ <p>SNAP</p>	Hours [23]
$\begin{array}{c} \text{NHCO}(\text{CH}_2)_2\text{CH}(\text{NH}_2)\text{COO}^- \\ \\ \text{ON}-\text{SCH}_2\text{CH} \\ \\ \text{CONHCH}_2\text{COO}^- \end{array}$ <p>GS-NO</p>	Days [23]

Table 2
Reaction of DEA/NO with Two Cell Types: Effect on Thiol and Zinc Distribution

[Zn] $\mu\text{M}^{a,b}$	NO ^c	Proteome ^{d,e}		MT ^{d,e}		LMW ^{d,e}	
		SH, nmol	Zn, nmol	SH, nmol	Zn, nmol	SH, nmol	Zn, nmol
0	–	1230±20	72±8	42±2	4 (8) ^f	480 ^g	18±1
0	+	900±40	67±1	35±3	3 (11)	330±10	20±2
80	–	1290±10	88±3	195±5	41 (67)	435±5	22 ^g
80	+	990 ^g	60±2	160 ^g	42 (52)	340±10	19±1
0	–	1615±45	57±5	54±1	5 (11)	425±15	30±2
0	+	1375±85	50±6	48±3	3 (8)	355±5	19±1
80	–	1440	68	600	65	440	27
80	+	1195±25	57 ^g	280	66 (82)	385±5	32±4

^aTop four rows: TE671 cells treated with 0 and 80 μM Zn^{2+} for 1 day.

^bBottom four rows: LLC-PK₁ cells incubated in the same way.

^c+/-NO indicates with or without DEA/NO treatment. Cells were exposed to 0 or 0.45 mM DEA/NO for 1 hour after Zn^{2+} treatment before cell harvesting. Homogenate was divided in 2; one-half was treated with Cd^{2+} and supernatants from both were chromatographed over Sephadex G-75. Except for MT Zn^{2+} values, the tabulated figures generally represent the average of the results of the two chromatographic runs $\pm$ the range of the results.

^dProteome, MT and LMW represent the three bands in the Sephadex G-75 size exclusion chromatography pattern shown in Figure 9.

^eSulphydryl and the metal contents were obtained by peak integration from the size exclusion chromatography and the result were normalized to 10^8 cells. Sulphydryl contents were determined by DTNB assay; The Zn^{2+} concentration was determined by atomic absorption spectroscopy.

^fIn parenthesis, the amount of $\text{Cd}^{2+} + \text{Zn}^{2+}$ associated with the MT band after Cd^{2+} incubation with homogenate as in (^c).

^gTwo averaged values are identical.