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

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The reactivity of Zn_7- and Cd_7-metallothionein (MT) with *S*-nitrosopenicillamine (SNAP), *S*-nitrosoglutathione (GSNO), and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA/NO) was investigated to explore the hypothesis that metallothionein is a signficant site of cellular reaction of nitric oxide or NO compounds. Zn_7-MT reacted with SNAP or GSNO only under aerobic conditions and in the presence of light, which stimulates the decomposition of *S*-nitrosothiolates to NO. Zn^{2+} is released, and protein thiols are modified. DEA/NO, which degrades spontaneously to release NO, also reacted with Zn_7-MT only when oxygen was present. Anaerobically, DEA/NO reacted with Zn_7-MT in the presence of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, which converts NO to NO_2. Glutathione competed effectively with Zn_7-MT for reactive nitrogen oxide species in reaction mixtures. Reaction of Cd_7-MT with SNAP also required oxygen and light to react. In this case, only a fraction of the Cd^{2+} bound to Cd_7-MT was displaced by SNAP. Apo-metallothionein was much more reactive with SNAP and DEA-NO than Zn_7- or Cd_7-MT. TE671 and LLC-PK_1 cell lines were incubated with DEA/NO to examine the role that MT might play in the cellular reactions of this NO donor compound. Incubation of cells with 0−80 μM Zn^{2+} for 24 h resulted in progressively increasing concentrations of Zn-unsaturated MT. One hour of cellular exposure to a range of DEA/NO concentrations followed by 24 h of incubation caused no evident acute toxicity at less than 0.45 mM. Preinduction of MT did not alter this response. The effects of DEA/NO on proteomic, metallothionein, and low molecular weight (LMW) thiol pools, including glutathione (GSH), were measured. Substantial fractions of the proteomic and LMW thiol pools underwent reaction with little dislocation of Zn^{2+}. In addition, one-third of the MT thiol pool reacted without labilizing any of the bound Zn^{2+}. These results demonstrated that it was free thiols associated with MT that reacted with DEA/NO not those bound to Zn^{2+}. Moreover, under the conditions of the experiments, DEA/NO reacted with the spectrum of cellular thiols in proportion to their fraction in the cytosol and did not preferentially react with MT sulfhydryl groups.

Introduction

Mammalian metallothionein (MT[1]) is a small, thiol rich protein that binds multiple metal ions in two metal−thiolate clusters (*1*). Numerous studies, including ones with MT knockout mice, demonstrate that the presence of MT in cells protects them from excessive exposure to metal ions such as Cd^{2+} (*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 the hydroxyl radical (*3, 5*).

According to NMR and X-ray crystallographic studies, Cd_7-MT and Cd_n,$Zn_{(7-n)}$-MT contain two domains, each organized about a metal−thiolate cluster with stoichiometries of $M(Cd/Zn)_4S_{11}$ (C-terminal α-domain) and $M(Cd/Zn)_3S_9$ (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 the thiolate sulfurs is substantially reduced (*8*). Still, significant solvent exposure remains providing 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

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[1] Abbreviations: 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)-diazenolate 2-oxide; DETA/NO, 1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-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-tetramethylimidazoline-1-oxyl 3-oxide; RS-NO, general nitrosyl thiolate; MT, metallothionein; Zn-MT, metallothionein with focus on sites that have bound Zn^{2+}; SH, thiol or sulfhydryl group; MTT, thiazolyl blue tetrazolium bromide; SNAP, *S*-nitrosoacetylpenicillamine; SNOC, *S*-nitrosocysteine; Zn_7-MT, fully Zn^{2+} saturated metallothionein; zincon, 2-carboxy-2-hydroxy-5-sulfoformazyl-benzene sodium salt.

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normal and knockout 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 Cd^{2+} 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 Zn^{2+} 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_n,$Zn_{(7-n)}$-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 Zn^{2+} (*15, 16*). Although the formation of S-nitroso-groups could not be detected in the absence of O_2, the characteristic absorbance of this species was immediately detected once the system was opened to air (*15*). In another experiment, using Cd_7-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 Cd_7-MT (*17*). Finally, it has been observed that one of the less common metallothionein isoforms, 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 Zn_7- and Cd_7-MT. We also considered the possible products of these reactions and the comparative reactivity of Zn_7-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 Zn^{2+} 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-PK_1, tubular cells from pig kidney.

Materials and Methods

Materials. Rabbit liver Zn_7-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 Zn^{2+} to SH ratio was typically 2.9. The metal-free form of MT, apo-MT, was prepared by acidification of Zn_7-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. Cd_7-MT was prepared by reconstituting apo-MT with Cd^{2+} 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 Da were purchased from Bio-Rad. Spectrophotometric measurements were carried out with a Beckman Coulter DU 640 UV−vis spectrophotometer.

Zinc Release Assay. Zn_7-MT and NO related compounds were mixed and incubated in cuvettes. After a given period of time, zincon, which does not react with native Zn_7-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 the zinc−zincon complex at 620 nm (ε_{620} = 23,200 M^{-1} 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 the reaction. This discontinuous method of kinetic analysis was used to measure free Zn^{2+} 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 Zn_7-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 Zn^{2+} to bind to MT, was the difference between the free Zn^{2+} at time *t* and that at time zero.

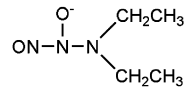
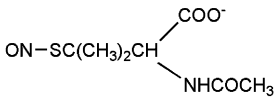
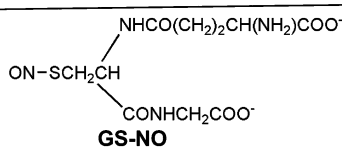
Light Effect upon the Reactions of MT with NO Donor Compounds. The role of light in the reactions of Zn_7- or Cd_7-MT with NO donor compounds was studied by conducting parallel sets of experiments in the dark and in the presence of light provided by two Philips light tubes (TYPE F32T8/TL735) which were positioned 1 m above the reaction cuvettes. Zinc release was assayed as described above.

Effect of PTIO in the Reaction of Zn_7-MT with DEA/NO: Use of Spin Columns to Separate Metal Ions from Metallothionein. Under anaerobic conditions, 6 μM Zn_7-MT was reacted with 300 μM DEA/NO for 15, 30, and 80 min 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 Zn^{2+} content of the solution that passed through the membrane during centrifugation. As a control, >90% of 6 μM Zn_7-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% CO_2 environment as previously described (*24*). Bovine fetal serum concentration was 3.3% for maintenance cell culture and 5% for experiments. LLC-PK_1 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 μM Zn^{2+}, there was no discernible 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 μL 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.

Table 1. NO Reagents and Their Properties

Compound	NO Release, $t_{1/2}$
 **DEA/NO**	20 minutes, This work
 **SNAP**	Hours [23]
 **GS-NO**	Days [23]

Chromatography of TE671 and LLC-PK$_1$ Cells. Cells were rinsed, harvested, and homogenized with an ultrasonicator. The homogenate was then centrifuged at 50,000*g* × 20 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 Cd^{2+} displacement of the MT bound Zn^{2+} and metal-free SH groups associated with Cd^{2+}. Then the SH/(Zn^{2+} + Cd^{2+}) content of the peak was calculated using the value in parentheses for Zn^{2+} + Cd^{2+} listed in Table 2. Total MT was taken to be (Zn^{2+} + Cd^{2+})/7; total SH was (Zn^{2+} + Cd^{2+}) × 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 Zn_7-MT.

Data Analysis. All chemical experiments were carried out at least 3 times. Where fixed time measurements of reaction progress were made, averages ± 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 Zn_7-MT with S-nitroso-compounds as well as NO and its oxidation products (Table 1). With SNAP and GS-NO, the transnitrosation reactivity of Zn_7-MT was assessed (reaction 1).

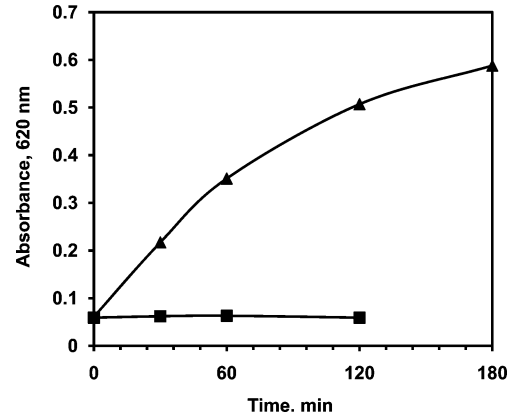


Figure 1. Oxygen requirement of the reaction of Zn_7-MT with SNAP. Conditions: 6 μM Zn_7-MT and 300 μM SNAP plus 200 μM zincon in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C. The maximum possible absorbance is approximately 1.0 (▲, aerobic; ■, anaerobic).

$$\text{MT-S}^- + \text{RS-NO} \rightleftarrows \text{MT-S-NO} + \text{RS}^- \qquad (1)$$

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*):

$$\text{RS-NO} \rightarrow \text{NO}^{\cdot} + \text{RS}^{\cdot} \qquad (2)$$

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 sulfhydryl groups with NO (anaerobic conditions) or NO, NO_2, and N_2O_3 (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 noncysteine 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-PK$_1$ pig kidney proximal tubule cells.

Reaction of Zn_7-MT with SNAP-Requirement for Oxygen. The reaction of 6 μM Zn_7-MT with 300 μM SNAP was studied as a function of the presence of light and oxygen. Zn^{2+} release was detected by an increase in absorbance of the Zn-zincon complex at 620 nm (Figure 1). In aerobic reaction mixtures exposed to light, more than 20% of the metallothionein-bound Zn^{2+} became available for zincon chelation (~8 μM Zn^{2+}) within 60 min; 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 Zn^{2+} release (data not shown). In contrast, there was no observable formation of Zn-zincon under anaerobic conditions in the presence of light, indicating that Zn_7-MT did not undergo a reaction with NO, the decomposition product of SNAP, in the absence of O_2 (Figure 1). Nevertheless, light-exposed SNAP, itself, was degraded in the absence or presence of oxygen. The same O_2 requirement for the effective liberation of Zn^{2+} from Zn_7-MT

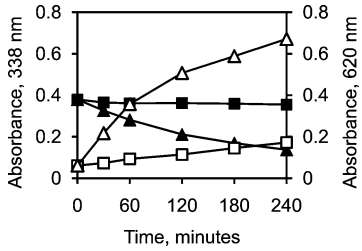


Figure 2. Light dependence of SNAP decomposition and Zn^{2+} release. Conditions: 6 μM Zn_7-MT and 300 μM SNAP plus 200 μM zincon in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C under aerobic conditions. The absorbance decrease at 338 nm due to the loss of the S-nitroso bond of SNAP in the reaction mixture (■, dark; ▲, light) and the Zn^{2+} release, measured by the formation of the Zn-zincon chromophore at 620 nm (□, dark; △, light).

was defined in the reactions of Zn_7-MT with GSNO and DEA/NO as described below. This indicated that the O_2 requirement is a general property of these reactions of Zn_7-MT with NO donor compounds.

Light As a Factor in the Reaction of Zn_7-MT with SNAP. Light sensitivity is a common property for many NO donor compounds including SNAP (*29, 30*) Using the same reaction conditions as those in Figure 1, very little zinc release was observed when light was excluded from the aerobic reaction mixture (Figure 2). During the first 2 h, only about 3% of the Zn^{2+} 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. A hypothesis consistent with these results is that an *S*-nitrosothiolate interchange reaction occurred as follows, which displaced Zn^{2+} from MT without altering the total concentration of RS-NO:

$$\text{RS-NO (SNAP)} + \text{Zn-S-MT} \rightleftarrows \text{RS}^- + \text{Zn}^{2+} + \text{MT-S-NO} \qquad (3)$$

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 2 h as shown in Figure 1.

Reaction of GSNO with Zn_7-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 Zn_7-MT with GS-NO were similar to those with SNAP except that the reactions occurred more slowly. Thus, after 2 h of aerobic reaction of 6 μM Zn_7MT and 300 μM GS-NO in the presence of light, only 5% of the total Zn^{2+} was released. Extending the reaction time to 5 h increased the extent to 13%. When the concentration of GS-NO was doubled to 600 μM, Zn^{2+} release increased to 8% in 2 h and 18% in 5 h. No Zn^{2+} was released in the absence of O_2 (data not shown) or light.

Reaction of DEA/NO with Zn_7-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 min at pH 7.4 and 25 °C. As in other reactions,

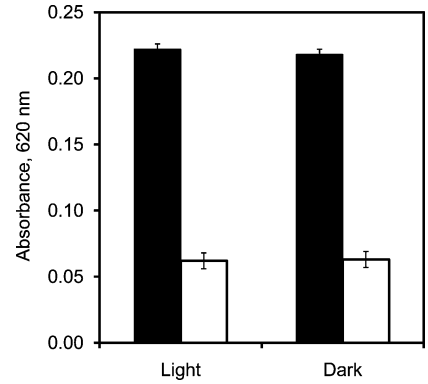


Figure 3. Reactions of DEA/NO with Zn_7-MT. Conditions: 6 μM Zn_7-MT and 300 μM DEA/NO plus 200 μM zincon in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C for 1 h. Reactions were conducted in either light or darkness. Effect of O_2 on the release of free zinc from MT. Comparison of the Zn-zincon absorbance under aerobic and anaerobic conditions (■, aerobic; □, anaerobic).

6 μM Zn_7-MT was allowed to react with 300 μM DEA/NO (Figure 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 (Figure 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 (Figure 1), the rate of reaction was not dependent on a photochemical event during the 1 h 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 Zn_7-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 Zn_7-MT with DEA/NO. Experiments were conducted to probe the nature of the reactive species in the aerobic reaction of DEA/NO with Zn_7-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).

$$2\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2 \qquad (4)$$

$$\text{NO}_2 + \text{NO} \rightarrow \text{N}_2\text{O}_3 \qquad (5)$$

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*):

$$\text{NO} + \text{PTIO} \rightarrow \text{NO}_2 + \text{PTI} \qquad (6)$$

Spin-column chromatography was used to evaluate the labilization of Zn^{2+} from Zn_7-MT during its reaction with DEA/NO in the absence and presence of PTIO (Figure 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

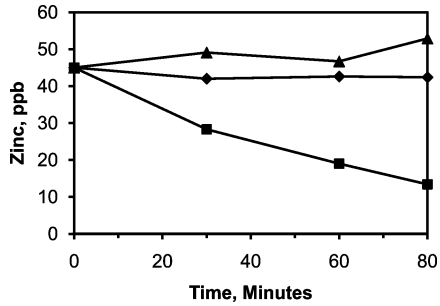


Figure 4. Effect of PTIO in the reaction of DEA/NO with Zn_7-MT. Conditions: 6 μM Zn_7-MT, 300 μM DEA/NO, and 300 μM PTIO in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C under aerobic conditions. At intervals, the reactions of Zn_7MT with DEA/NO (▲), PTIO (■), and DEA/NO+PTIO (♦) were separated with spin column chromatography and the separated free Zn^{2+} concentration measured by ICP-MS after 60-fold dilution.

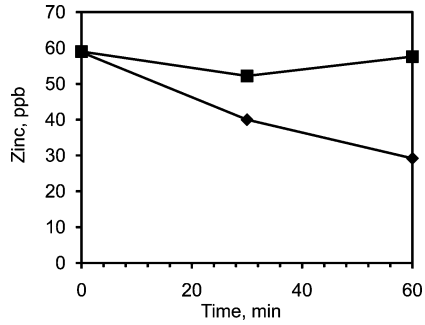


Figure 5. Properties of the product of NO and PTIO with Zn_7-MT: the effect of DTT. Conditions: 6 μM Zn_7-MT, 300 μM DEA/NO, and 300 μM PTIO in 50 mM Tris-Cl buffer, at pH 7.4 and 25 °C under anaerobic conditions. After 30 and 60 min, 0.5 mM DTT was added to the mixture. After a 5 min incubation, the mixture was separated with a spin column and the Zn measured by ICP-MS (■, with DTT reduction; ▲, without DTT reduction).

Zn_7-MT and PTIO. In contrast, when Zn_7-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 Zn_7-MT. In this experiment, 300 μM PTIO actually functioned much better than O_2 in air (~250 μM) as an oxidizing agent for NO.

The product of the anaerobic reaction of Zn_7-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 Figure 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 Zn_7-MT with SNAP in the Presence and Absence of GSH. The reaction of Zn_7-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 Zn_7-MT with SNAP (Figure 6). Similar inhibition of Zn^{2+} release by GSH in the reaction of Zn_7-MT with DEA/NO was also observed (data not shown).

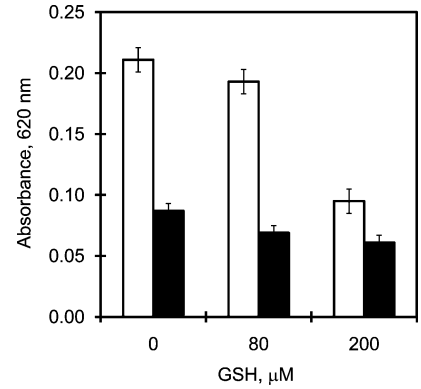


Figure 6. Effect of GSH on the reaction of SNAP with Zn_7-MT under aerobic conditions. Conditions: 6 μM Zn_7-MT and 300 μM of SNAP in 50 mM Tris-Cl buffer at pH 7.4 at 25 °C in the presence or absence of 80 or 200 μM GSH for 1 h. Zn^{2+} release was measured by the formation of the Zn-zincon chromophore at 620 nm (■, dark; □, light).

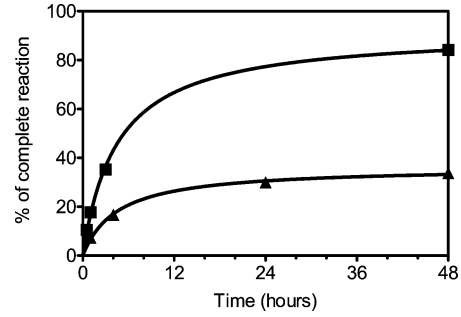


Figure 7. Reactions of SNAP with Zn_7-MT and Cd_7-MT. Conditions: 300 μM SNAP and 6 μM Zn_7-MT or 6 μM Cd_7MT in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C under aerobic conditions. The release of metal from MT was followed spectrophotometrically with 200 μM zincon (Zn) at 620 nm or 200 μM PAR (Cd) at 486 nm (ε_{486} of Cd-PAR = 4.2×10^4 M^{-1} cm^{-1}) (■, Zn_7MT; ▲, Cd_7MT).

Reaction of Cd_7-MT with SNAP and DEA/NO. The reactions of Zn_7-MT and Cd_7-MT with SNAP are compared in Figure 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% upon the basis of 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 Cd_7-MT; repeated additions of 300 μM DEA/NO could not push the reaction further. The extent and limit of the Cd_7-MT reaction with DEA/NO was consistent with only the Cd_3-β domain undergoing the reaction. In contrast, both domain clusters of Zn_7-MT underwent the 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 overproducing 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 (Figure 8). Unlike the comparable reactions of Zn_7-MT, there was no O_2 or light dependence of 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.

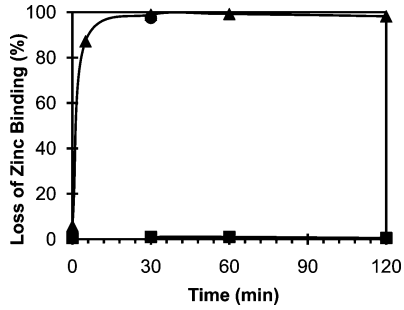


Figure 8. Reactions of apo-MT with NO compounds. Conditions: 4 μM apo-MT and 300 μM SNAP or DEA/NO in 50 mM Tris-Cl buffer at pH 7.4 and 25 °C under anaerobic conditions. Free Zn^{2+} in solution was measured with 200 μM zincon after the addition of 40 μM Zn^{2+} to the reaction mixture to bind to the remaining apo-MT. For comparison, the same procedure was applied to 6 μM Zn_7-MT with 300 μM SNAP. The data is presented as the percent of the possible absorbance at 620 nm (■, Zn_7MT with SNAP; ▲, Apo-MT with SNAP; ●, Apo-MT with DEA/NO, 30 min point only).

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 that of other major pools of thiol groups with DEA/NO in two cell lines under control and Zn^{2+} induced conditions that elevated the concentration of metallothionein.

Effect of DEA/NO on TE671 and on LLC-PK$_1$ 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-PK$_1$ cells as the DEA/NO concentration was increased above 0.45 mM. Preincubation with Zn^{2+} 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 Zn^{2+} associated with the proteome, metallothionein, and LMW/glutathione pools (Figure 9a). Both thiol and Zn^{2+} 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 kDa, eluted at the position centered on fraction 30, exhibiting a small TNB absorbance band or shoulder between the other two peaks. Control LLC-PK$_1$ cells displayed a qualitatively similar profile, with distinct proteome and LMW bands of sulfhydryl groups and Zn^{2+} and little evidence of MT between them.

Peak integrations for the TE671 chromatographic peaks showed that per 10^8 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 Zn^{2+} in the MT part of the chromatographic profile do not necessarily represent MT, we conducted a Cd^{2+} saturation experiment 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 Zn^{2+} quantification among the components of LLC-PK$_1$ cell supernatant.

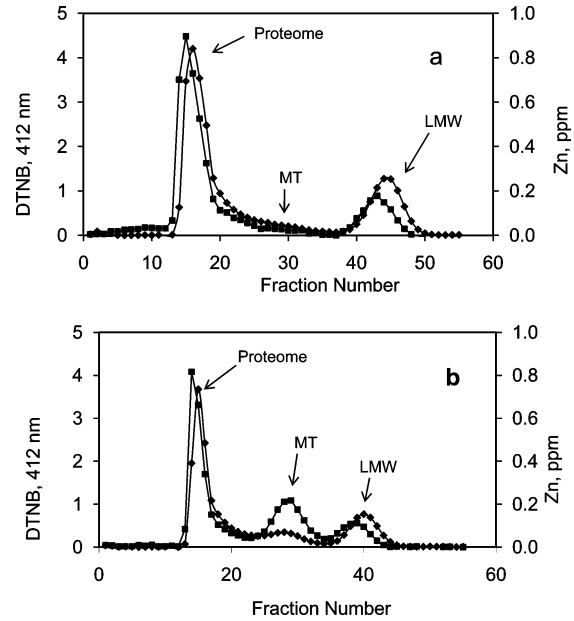


Figure 9. Sephadex G-75 gel filtration column chromatography separation of TE671 cell supernatant Control cells (a) and cells incubated with 80 μM $ZnCl_2$ for 24 h (b) were chromatographed and the fractions tested for zinc (■) using atomic absorption spectrophotometry and SH (♦) using the DTNB colorometric assay which measures the absorbance of the reagent at 412 nm. The three major zinc peaks, Zn-proteome, MT, and LMW are indicated.

The presence of a sizable low molecular weight Zn^{2+} pool was notable. It precedes the thiol/glutathione sulfhydryl peak and thus does not represent free Zn^{2+} or a Zn−glutathione complex. Its identity is unknown but is under investigation.

MT Induction by Zn^{2+}. MT content was clearly boosted in both sulfhydryl and Zn^{2+} when the TE671 cells were preincubated with 80 μM Zn^{2+} for 24 h. Discrete MT peaks were revealed by both the DTNB and the Zn^{2+} analyses (Figure 9b). The thiol and Zn^{2+} contents of the proteome and LMW pools were unaffected by 24 h exposure to Zn^{2+}. In contrast, the MT thiol content was boosted more than 5 fold, increasing from 42 nmol to 195 nmol. The Zn^{2+} content in the MT band also increased from 4 to 41 nmol. The MT SH/Zn^{2+} of 4.8 indicated that much of the protein was not saturated with Zn^{2+}. This was confirmed with the Cd^{2+} saturation experiment that showed that the SH/Zn^{2+} + Cd^{2+} ratio was 2.9, indicating that the total SH content of the band was assignable to MT.

In the case of LLC-PK$_1$ cells, incubation for 24 h with 80 Zn^{2+} μM did not impact the proteome, LMW thiol, or Zn^{2+} concentrations. It did, however, greatly increase the MT thiol content from 54 to 600 nmol/10^8 cells. Although the corresponding Zn^{2+} concentration was elevated 13 times, the SH/metal ion ratio of the protein was 9.2, indicating 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/10^8 cells from the proteome pool when compared with that of 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. On the basis

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

		proteome[d,e]		MT[d,e]		LMW[d,e]	
[Zn] (μM)[a,b]	NO[c]	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

[a] Top four rows: TE671cells treated with 0 and 80 μM Zn^{2+} for 1 day. [b] Bottom four rows: LLC-PK_1 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 h after Zn^{2+} treatment before cell harvesting. Homogenate was divided in two; 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 ± the range of the results. [d] Proteome, MT, and LMW represent the three bands in the Sephadex G-75 size exclusion chromatography pattern shown in Figure 9. [e] Sulfhydryl and the metal contents were obtained by peak integration from the size exclusion chromatography, and the results were normalized to 10^8 cells. Sulfhydryl contents were determined by DTNB assay. The Zn^{2+} concentration was determined by atomic absorption spectroscopy. [f] In parentheses, the amount of Cd^{2+} + Zn^{2+} associated with the MT band after Cd^{2+} incubation with homogenate as in c is shown. [g] Two averaged values are identical.

of the Zn^{2+} content, 76 and 119 nmol SH/10^8 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 noninduced 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 10^8 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 assocated with Zn_7-MT. That the sulfhydryl groups involved stemmed from MT protein was confirmed in the Cd^{2+} saturation experiment, which showed that the SH/(Zn^{2+} + Cd^{2+}) ratio in the MT pool was 3.1 after DEA/NO treatment. This result was consistent with 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 1 h on LLC-PK_1 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-PK_1 cells caused significant thiol loss from both the proteome and LMW bands. In the experiments shown, per 10^8 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 SH/Zn^{2+} ratio of 9.2, much of the band contained MT without bound Zn^{2+}. About 410 nmol of this thiol pool was 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 Zn_7-MT. Nevertheless, its SH/Zn^{2+} ratio remained above that for Zn_7-MT at 4.2, indicating that metal-free sulfydryl groups remained in the MT pool. This was confirmed with the Cd^{2+}-saturation assay, in which the resultant SH/(Zn^{2+} + 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).

$$\text{RS-NO} \rightarrow \text{RS}^{\bullet}(1/2\text{RSSR}) + \text{NO}^{\bullet} \quad (7)$$

$$\text{RS-NO} + h\upsilon \rightarrow \text{RS}^{\bullet}(1/2\ \text{RSSR}) + \text{NO}^{\bullet} \quad (8)$$

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

$$\text{RS-NO} + \text{R}'\text{S}^{-} \rightarrow \text{R}'\text{S-NO} + \text{RS}^{-} \quad (9)$$

$$\text{RS-NO} + \text{R}'\text{S}^{-} \rightarrow \text{R}'\text{S-SR} + \text{NO}^{-} \quad (10)$$

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 (Figures 1 and 2). Similarly, rapid decay of DEA/NO in the light or dark but only in the presence of O_2 also destroyed the MT Zn-thiolate clusters and released Zn^{2+} (Figure 3). Hence, it was evident that further reaction of NO was necessary to produce a species that was reactive with Zn_7-MT.

The lack of much direct reactivity of SNAP or GS-NO with Zn_7-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 Zn_7-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 Zn_7-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 (Figure 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 nonrate limiting. S-nitroso-species may be produced as follows in reactions 4, 5, and 11:

$$N_2O_3 + \text{MT-(S)}_2\text{-Zn} + H^+ \rightarrow \text{MT-(SH)(SNO)} + NO_2^- + Zn^{2+} \quad (11)$$

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

$$\text{MT-(SH)(SNO)} \rightarrow \text{MT-(SS)} + NO^- + H^+ \quad (12)$$

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 (Figure 3). Another experiment demonstrated that the substitution of an oxygen atom donor, PTIO, for O_2 in the DEA/NO reaction with Zn_7-MT results in a relatively rapid degradation of the Zn-thiolate clusters (Figure 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 Zn_7-MT than NO_2 (reactions 9 and 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:

$$\text{MT-(S)}_2\text{-Zn} + 2NO_2 \rightarrow \text{MT-(SS)} + Zn^{2+} + 2NO_2^- \quad (13)$$

The modified metallothionein that resulted from the reaction of NO_2 with Zn-MT could be wholy restored to its metal binding form by incubation with DTT (Figure 5). The simplest explanation is that the initial reaction oxidized the sulfhydryl groups of MT to disulfides, leading to the dissociation of Zn^{2+} (reaction 14). Then, DTT reduced the oxidized protein through a thiol-disulfide interchange reaction:

$$\text{MT-(SS)} + \text{DTT-(SH)}_2 \rightarrow \text{MT-(SH)}_2 + \text{DTT-(SS)} \quad (14)$$

The experiments cannot rule out the possibility that DTT competed successfully for MT-bound S-nitroso groups. Nevertheless, since the reactive species appeared to be NO_2 not NO, the formation of MT-S-NO did not seem likely.

The capacity of SNAP and DEA/NO to react with Zn_7- and Cd_7-MT was compared in order to follow up the earlier observations that DEA/NO only liberates Cd^{2+} from the Cd_3S_9 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 apoprotein is identical for each reaction, a thermodynamic difference could only reside in the dissociation step. However, each metal−thiolate cluster of Cd_7-MT binds metal ions several orders of magnitude more strongly than those in Zn_7-MT (*38*). Thus, the difference in behavior of the Zn_4S_{11} and Cd_4S_{11} clusters cannot result from differences in metal−thiolate bond strength; otherwise, the same lack of reactivity would be anticipated for the Cd_3S_9 cluster. The alternative is that a major kinetic barrier blocks the reaction of Cd_4S_{11} but is absent in the case of Zn_4S_{11}. Although quantitative differences in reactivity of the α- and β-domains with reagents such as EDTA and DTNB have been noted on the basis of 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 Zn_7-MT in the presence and absence of GSH. Competing 80 or 200 μM GSH against 120 μM MT thiol, we lowered the extent of Zn^{2+} release 37% and 55%, respectively. Thus, GSH is an effective competitor for reactive NO-species such as NO_2 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 Zn_7-MT would be the sole, let alone preferred, site of reaction of NO_2.

The reactions of apo-MT with SNAP or DEA/NO were strikingly faster than those with Zn_7- or Cd_7-MT (Figure 8). For example, the rate enhancement in the SNAP reaction that was realized by the substitution of apo-MT for Zn_7-MT was about 20-fold. Furthermore, like the reactions of GSH, those of apo-MT occurred in the dark and did not require O_2. 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.

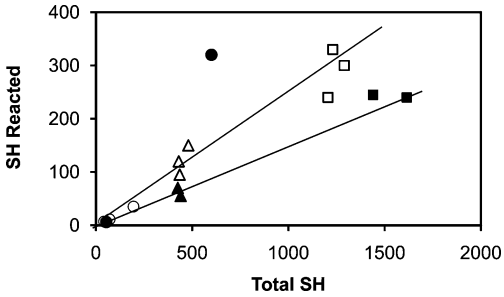


Figure 10. Cell thiol pools: relationship of SH reactivity to concentration. Data for TE671 (filled symbols) and LL-CPK$_1$ cells (open symbols) taken from Table 2 (■, □, proteome SH; ●, ○, MT SH; ▲, △, LMW (glutathione) SH).

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 Zn_7-MT, these observations support the hypothesis that NO compounds may target cellular apo-MT.

Overall, the reactivity of Zn_7-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-PK$_1$ cells (Table 2). Included among the reactive species were proteomic and low molecular weight thiols, the latter probably constituted primarily of glutathione. However, Zn_7-MT was not a significant target of DEA/NO at elevated concentrations of MT, including one involving LLC-PK$_1$ cells induced with 80 μM Zn^{2+}, 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-PK$_1$ cells (Table 2).

These results closely resemble the in vitro findings: MT sites with bound Zn^{2+} 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 NO_2 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 versus its total concentration from Table 2. In the case of TE671 cells, the linear character of the graph indicates 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 Zn^{2+} treated cells, less than half of the MT unbound thiol groups underwent the 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 that of other thiol pools.

About 8% of the reactive SH groups were derived from apoMT in TE671 cells incubated with 80 μM Zn^{2+}. This increased substantially to nearly 50% with LLC-PK$_1$ cells pretreated with 80 μM Zn^{2+}. In this case, when apo-MT was present in large concentrations in comparison to that of 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 the 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 the 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 alongside 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 as to 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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