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Sensor Specific Imaging of Proteomic Zn²⁺ with Zinquin and TSQ after Cellular Exposure to N-ethylmaleimide

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Abstract

The impact of the thiol binding reagent N-ethylmaleimide (NEM) on proteomic Zn²⁺ availability was investigated in rat glioma cells. Zinquin (ZQ) or TSQ, two related fluorescent sensors, were used to observe reactive Zn²⁺. Control cells contained proteomic Zn²⁺ but no detectable low molecular weight (LMW) Zn²⁺. With either sensor, basal cellular fluorescence emission centered near 470 nm, indicative of sensor-Zn-proteins. ZQ sequestered 13% of proteomic Zn²⁺ as Zn(ZQ)₂; TSQ reacted only with the Zn-proteome. NEM (100 μM) abolished LMW thiols, including glutathione (GSH) and lowered proteomic sulfhydryl content about 30%. In ZQ-treated cells, NEM exposure enhanced fluorescent intensity and the formation of Zn(ZQ)₂ (λ_{MAX}, 492 nm). Cells incubated with TSQ and NEM also displayed increased fluorescence without a spectral shift in wavelength maximum, consistent with increased formation of TSQ-Zn-protein adducts but not Zn(TSQ)₂. In neither experiment was Zn²⁺ lost from cells. NEM altered Zn²⁺ accessibility to sensors in membrane-nuclear and cytosolic fractions, but Zn(ZQ)₂ was only generated in the cytosol. Similar results were obtained when cell supernatant replaced cells. In contrast, when isolated proteome was reacted with ZQ and 100 μM NEM in the absence of GSH, 70% of the proteomic thiols underwent reaction. As a consequence, most of the ZQ-Zn-protein adducts were converted to Zn(ZQ)₂. Substituting TSQ for ZQ, only increased TSQ-Zn-proteins were observed. Evidently, the results of imaging cells with Zn²⁺ sensors are dependent upon the specific chemical properties of the sensors and can only be understood after detailed chemical analysis.

Introduction

Fluorescent sensors have been increasingly used to observe the distribution and trafficking of cellular Zn²⁺.^{1–3} Among the most widely utilized are the quinoline toluene sulfonamide compounds TSQ and Zinquin.^{4–9} These are bidentate metal binding ligands that form two to one complexes with Zn²⁺.¹⁰

The common understanding of these and other Zn²⁺ sensors has been that as they undergo changes in fluorescence within cells, they image Zn²⁺.⁷ Cells contain little or virtually no free Zn²⁺.^{11–12} Instead, almost all of the cell's complement of Zn²⁺ is bound to discrete proteins.¹³ A small amount may associate with proteins or glutathione and other small molecules during transit.¹⁴ Taken as a whole, these Zn²⁺ binding sites (P) establish equilibria with Zn²⁺ according to the following generic reaction:

Depending on the equilibrium properties of a particular sensor and Zn-P complex in reactions 1 and 2, incubation of cells with sensor will result in the formation of some concentration of fluorescent Zn-sensor. Moreover, the sensor might be able to react directly through ligand substitution with Zn-P according to reaction 3:

Thus, the extent of formation of fluorescent product will also reflect the kinetics of the particular reactants. It is evident that various Zn^{2+} sensors with their particular chemical properties may access different Zn-proteins species and form differing amounts of fluorescent Zn-containing products.

Another reaction that sensors may undergo with Zn-P is adduct formation:

In the examples of TSQ and Zinquin, reaction 4 describes the predominant net reaction of these sensors in a variety of cell types.^{15–16} In forming such adducts, these fluorophores image an estimated 10–20% of total proteomic Zn^{2+} or Zn^{2+} bound within the proteome. This is a new understanding contrasting with the view that these probes react with cells via reaction 1 or possibly 3.

Having established how these reagents react with the native Zn-proteome, it was important to consider trafficking of Zn^{2+} under conditions that apparently alter its intracellular distribution. N-ethylmaleimide (NEM) is a cell permeant molecule that preferentially reacts with sulfhydryl groups and has been used repeatedly to alter intracellular thiol status.^{17–18} In this study, we reexamined reports that NEM mobilizes and rearranges Zn^{2+} in cells as previously measured by the fluorescent Zn^{2+} sensors Zinquin and FluoZin-3.^{19–21} Our intent was to understand the nature of the chemical species of Zn^{2+} that these sensors image.

Materials and Methods

Chemicals

Zinquin Ethyl Ester and Zinquin Acid were purchased from Enzo Life Sciences (Plymouth Meeting, PA). TSQ was purchased from AnaSpec (Fremont, CA). Probes (Figure 1A) were solubilized in DMSO and stored in the dark at 4° C. N-ethylmaleimide (Figure 1B) was solubilized in ethanol at a stock concentration of 200 mM and stored in the dark and used within 2 weeks. All other chemicals were purchased from Sigma-Aldrich or Fisher Chemical Companies.

Cell Culture

C6 rat brain glioma cells were purchased from the American Tissue Cell Culture. Cells were grown in F-12K culture media supplemented with 15% horse serum, 2.5 % fetal calf serum, 50 mg/L Streptomycin and 50 mg/L Penicillin G. Cultures were grown in the presence of 5% CO_2 /95% Air at 37 °C.

Toxicity of NEM

Cells were plated in complete medium at a concentration of approximately 1×10^5 cells/mL in 24-well plates overnight. Then, 0, 25, 50, 100, 150, or 200 μM NEM was added to the wells for 15, 30, 45 or 60 minutes. After exposure, the medium containing NEM was decanted and cells were washed 3X with 1 mL of Dulbecco's phosphate buffered saline (DPBS). One mL of complete medium containing 500 μM 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium, bromide (MTT) was added to the wells and allowed to incubate

for 4 hours at 37 °C.²² Then, 1 mL of acidified (0.04 N HCl) isopropanol was added to the wells in order to dissolve the newly formed formazan crystals. Absorbance at 570 nm and 630 nm was recorded and the difference in absorbance at the two wavelengths was used to calculate viability of dosed cells based on a comparison with unexposed cells.

Fluorescence spectrophotometry

Cells grown on 100 mm culture plates were washed 3X with cold DPBS and gently scraped into a DPBS suspension at room temperature using a rubber cell scraper. Fluorescence readings were taken using a Hitachi F-4500 fluorescence spectrophotometer. An excitation wavelength of 370 nm was used to record the emission spectra from 400–600 nm. The initial spectrum of unexposed cells was taken followed by exposure to 25 μ M ZQ_{EE} for 30 minutes before subsequent fluorescent readings. Cells were exposed to 100 μ M *N*-ethylmaleimide and the changes in emission spectrum were monitored for 20 minutes. All fluorescence spectra were recorded in arbitrary units at room temperature and corrected for basal autofluorescence.

Separations of Cell Lysates

Cells incubated with Zinquin $\pm$ NEM were collected via centrifugation at $640 \times g$ for 4 minutes at room temperature. Cells were resuspended in 1 mL of cold double distilled H₂O and sonicated for 60 pulses. Sonicate was then centrifuged at $47,000 \times g$ for 20 minutes at 4 °C. The supernatant was loaded onto a 0.7 cm $\times$ 80 cm Sephadex G-75 (GE Healthcare) column and eluted with degassed 20 mM Tris-Cl buffer pH 7.4 into 50 1 mL fractions. The fluorescence emission spectrum of each fraction was recorded. Sulfhydryl group content was then determined with Ellman's Reagent.²³ Specifically, 100 μ L of 10 mM 5,5-Dithiobis-2-nitrobenzoic acid (DTNB) were added to each fraction and allowed to incubate for 30 minutes before the absorbance at 412 nm was recorded. Using an extinction coefficient of $13,400 \text{ cm}^{-1} \text{ M}^{-1}$, the concentration of reactive thiol groups was determined. The concentration of Zn²⁺ in each fraction was determined by flame atomic absorption spectrophotometry.

Reaction of Zn-proteome with NEM

C6 cells were scraped, sonicated, and centrifuged as previously described. The supernatant was loaded onto a 1.5 cm $\times$ 15 cm Sephadex G-25 column and eluted with degassed 20 mM Tris-Cl pH 7.4. Initial fractions containing absorbance at 280 nm, indicative of protein, were pooled and Zn²⁺ was determined by flame atomic absorption spectrophotometry. Subsequently, 10 μ M C6 proteome (based on Zn²⁺) was reacted with 20 μ M ZQ_{ACID}/TSQ for 30 minutes. The proteome was then incubated with 100 μ M NEM for 30 minutes before being loaded onto a 0.7 cm $\times$ 40 cm Sephadex G-50 gel filtration column that was eluted with 20 mM Tris-Cl pH 7.4. Thirty 1 mL fractions were collected and fluorescence, [Zn²⁺], and thiol concentrations were determined.

Subcellular fractionation of C6 cells exposed to NEM

Subcellular fractionation was performed according to Petering et al. with modifications.²⁴ Briefly, twin aliquots of cells were exposed to ZQ_{EE} and one of them to NEM as described above. Cells were collected via centrifugation at $640 \times g$ for 4 minutes at room temperature. Cell pellets were resuspended in 3 mL of pre-chilled homogenation buffer (0.25 M sucrose, 15 mM potassium chloride, 1.5 mM magnesium sulfate, 1 mM 2-mercaptoethanol, 10 mM Tris-Cl pH 7.4). The resultant suspension was homogenized by hand using a pestle and centrifuged at $1000 \times g$ for 10 minutes at 4 °C. The pellet was saved as the nominal membrane-nuclear fraction. Supernatant was centrifuged at $10,000 \times g$ for 15 minutes at 4 °C. The pellet was saved as the putative mitochondrial fraction. Supernatant was centrifuged

again at $27,000 \times g$ at 4°C and the pellet was saved as the lysosome/peroxisome fraction. A final centrifugation was performed at $100,000 \times g$ for 40 minutes at 4°C , yielding the microsome pellet and the cytosol supernatant. All pellets were resuspended in 1 mL of homogenation buffer and the fluorescence spectra as well as the Zn^{2+} concentrations were recorded as previously stated.

Statistics

Experiments with different samples were carried out at least 3 times. Results are shown as averages $\pm$ standard deviation for n experiments.

Results

Toxicity of N-ethylmaleimide

To determine the cellular health of C6 cells after acute exposures to *N*-ethylmaleimide, the MTT assay was performed.²² As expected, cytotoxicity was dependent not only on the concentration of NEM but more importantly, the length of exposure (Figure 2). Incubations for more than 30 minutes began to significantly affect cellular viability. For this reason, experiments with intact C6 cells were not incubated in NEM for longer 30 minutes.

Fluorescence changes of cells exposed to NEM

The reaction of C6 cells with a hypothetical Zn^{2+} releasing agent, NEM was investigated with fluorescence spectrofluorimetry, using the Zn^{2+} fluorescent probe Zinquin to follow the course of reaction.¹⁶ Upon exposure of cells to ZQ_{EE} , the resulting fluorescence emission spectrum was centered at 468 nm, consistent with the formation of ZQ-Zn-protein ternary adducts.¹⁶ These cells were then treated with $100 \mu\text{M}$ NEM that was hypothesized to react with Zn^{2+} binding sites that contained thiolate ligands. As seen in previous studies, the fluorescence intensity increased over time, in this case by $37 \pm 10\%$ ($n = 3$) (Figure 3). To test for the release of protein bound Zn^{2+} as free Zn^{2+} , the ratio the emission intensities at 492 nm (λ_{MAX} of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$) and 468 nm (λ_{MAX} of ZQ-Zn-Protein) was recorded over time. This ratio increased steadily, such that after 20 min the spectral wavelength maximum had shifted from 469 nm to 479 nm. This result suggested the release of ligated Zn^{2+} from ZQ-Zn-proteins with thiol containing metal binding sites according to overall reaction 5 (Figure 3 (inset))

To investigate the basis of the fluorescence changes defined in this reaction, cells exposed to ZQ_{EE} and NEM were lysed and the supernatant separated by size exclusion gel filtration (Figure 4). The resulting chromatogram was compared to a control profile of C6 cells exposed to ZQ_{EE} alone. In the control, most of the fluorescence was associated with the proteome but some migrated as a low molecular weight species (Figure 4A). Upon reaction with NEM, much of the fluorescence was still confined to the proteomic region of the chromatograph (Figure 4A). These fluorescent fractions had emission profiles that were blue shifted to 467 nm, consistent with the formation of ZQ adducts with Zn^{2+} bound within the proteome.¹⁶ In addition, the pool of LMW fluorescence of the NEM-reacted cells grew compared to control cells. The emission spectra of these fractions from both control (not shown) and NEM-treated cells centered at 492 nm, consistent with the presence of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$.¹⁶ Integrating the fluorescence intensities of the two bands showed that the NEM exposed proteome experienced an increase in fluorescence of $16 \pm 8\%$ ($n=3$). In addition, the pool of LMW fluorescence of the NEM-reacted cells grew $30 \pm 11\%$ ($n=3$) compared to control cells treated only with ZQ .

Impact of NEM on Zn^{2+} distribution and SH content in cells treated with ZQ_{EE}

To determine how NEM affected the distribution of Zn^{2+} within the lysates, Sephadex G-75 chromatographic fractions from Figure 4 were analyzed for Zn^{2+} by flame atomic absorption spectroscopy. Chromatography of unexposed control cells demonstrated that all of the detectable Zn^{2+} was located in the proteomic fractions (Figure 4B). Upon incubation with ZQ_{EE} , a small peak of Zn^{2+} and SH was observed in the low molecular weight fractions (Figure 4B and C). We hypothesized that ZQ reacted with a small part of the Zn-proteome as in reaction 2 to generate $\text{Zn}(\text{ZQ})_2$. After exposure to NEM, only a small reduction of Zn^{2+} in the Zn-proteome of $9 \pm 4\%$ ($n=3$) was detected versus control of cells solely exposed to Zinquin (Figure 4B). This small reduction of Zn^{2+} in the proteome (2.2 ± 0.9 nmoles) was recovered in the LMW pool. No Zn-metallothionein was observed in the 10 kDa region of the chromatogram (Fractions 20–30). Thus, incubation with ZQ and NEM mobilized some but not much Zn^{2+} into the low molecular weight region of the chromatogram.

Thiol concentration was determined using Ellman's reagent.²³ NEM forms a covalent bond with reactive thiols (Reaction 5); therefore, this product should not be detected by the SH assay.¹⁷ The sulfhydryl content of cells reacted with $\text{ZQ}_{\text{EE}} \pm \text{NEM}$ is shown in Figure 4C. After the treatment with NEM, the LMW sulfhydryl pool was completely depleted (arrow). This pool consists mainly of glutathione, a glycine-cysteine-glutamate tripeptide that plays a major role in oxidative stress.²⁵ In addition to the loss of the glutathione pool, the thiol content of the proteome decreased $29 \pm 5\%$ ($n=3$). These data show that NEM underwent widespread reaction with intracellular thiols, both GSH and cysteinyl residues within the proteome. Evidently, the reaction of NEM with proteomic SH groups mobilized Zn^{2+} according to reaction 5.

Reaction of cells with NEM monitored with TSQ

The experiments involving ZQ were repeated with a related quinolosulfonamide probe, TSQ, to determine whether an analogous probe would yield similar findings. Upon exposure of cells to TSQ, the emission profile was centered at 475 nm (Figure 5). This is consistent with our previous study that reported on the reaction of C6 cells with TSQ.¹⁵ Once cells were reacted with NEM, the fluorescence intensity increased $28 \pm 6\%$ ($n=3$) over 20 min. However, unlike Zinquin-exposed cells, the ratio of intensities of free $\text{Zn}(\text{TSQ})_2$ species (490 nm) and that of TSQ-Zn-Protein (475 nm) stayed nearly constant throughout this period after the spectrum initially shifted to a wavelength maximum of 477 nm (Figure 5 inset).¹⁵ Since TSQ binds more weakly to Zn^{2+} than Zinquin, it was hypothesized that TSQ associated with NEM-modified Zn^{2+} binding sites to form adducts whereas Zinquin chelates Zn^{2+} from at least some of the NEM reacted Zn-proteins to form $\text{Zn}(\text{ZQ})_2$ (Reaction 6):

Fluorescence, Zn^{2+} and SH groups in cells exposed to NEM in the presence of TSQ

To probe whether free $\text{Zn}(\text{TSQ})_2$ was formed when cells were exposed to NEM, cell lysates were again separated by gel filtration (Figure 6). After fractionation, all the fluorescence remained within the proteomic fractions (Figure 6A). The emission maximum of this region was centered at 468 nm, indicative of TSQ-Zn-protein adducts and not free $\text{Zn}(\text{TSQ})_2$.¹⁵ Furthermore, no fluorescence, thiol groups, or Zn^{2+} was detected in the LMW region of the chromatograph, consistent with the pattern of NEM reactivity described with ZQ as well as the inability of TSQ to undergo reaction 3 with Zn-proteome (Figures 6B and C). We attribute this difference to TSQ's lower affinity for Zn^{2+} than ZQ. As in cells, the fluorescence intensity of the proteomic region increased $30 \pm 18\%$ ($n=3$) versus control. Taken together, these data suggest that thiolate reactions with NEM opened up a pool of

reactive Zn^{2+} from the proteome that both TSQ and Zinquin sense. Zinquin chelates some Zn^{2+} to produce the free $\text{Zn}(\text{ZQ})_2$ complex, whereas TSQ only formed additional TSQ-Zn-protein adducts.

Reaction of the Zn-proteome with NEM

Experiments were performed to help understand how NEM affects the Zn-proteome in relation to its reactivity with Zinquin and TSQ. The isolated Zn-proteome was reacted with ZQ_{ACID} followed by exposure to 100 μM NEM for 30 minutes. In a parallel control, the Zn-proteome was reacted only with ZQ_{ACID} . Sephadex G-50 chromatography of the Zn-proteome mixed with Zinquin showed that almost all of the fluorescence remained within the proteomic region (λ_{MAX} , 467 nm) together with nearly all of the Zn^{2+} (Figure 7A). In addition, there existed a small but noticeable LMW pool of fluorescence and Zn^{2+} (λ_{MAX} , 492 nm).

The NEM reaction mixture was also separated by gel filtration (Figure 7B). In this case, there existed two significant pools of fluorescence. Some fluorescence was still located in the proteomic region (λ_{MAX} , 470 nm) but was greatly diminished compared to the control. The majority of the fluorescence resided in the low molecular weight region of the chromatogram. This pool had an emission profile centered at 492 nm, characteristic of a $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ complex. Analysis of the Zn^{2+} concentrations showed that 25% of the Zn^{2+} eluted in the LMW region (3.4 of 12.8 total nmoles of Zn^{2+}). The sulfhydryl content of the NEM-reacted proteome was diminished by the approximate amount of NEM added (100 nmoles NEM; 166 nmoles of reactive thiols reduced to 54 nmoles after reaction). These data indicated that NEM reacted with sulfhydryl ligands, some of which were coordinated to Zn^{2+} in the proteome. In turn, this new pool of labile Zn^{2+} reacted with Zinquin to form $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$. Moreover, since the proteome displayed relatively little fluorescence after exposure to NEM, it is hypothesized that most of the original ZQ-Zn-protein ternary complexes were coordinated by sulfhydryl containing proteins that underwent reaction with NEM. The quantitative difference between the outcome of this reaction and that of whole cells, in which much less modification of proteomic SH groups was observed, was apparently due to the presence of GSH in cells. It competed successfully for much of the NEM, thus sparing thiol groups in the proteome.

This interpretation gained support from an experiment in which 50 nmol of glutathione was added back to the proteome before reaction with 100 nmol NEM (Figure 7C). In comparison with the reaction of proteome alone with NEM, the addition of GSH greatly expanded the concentration of SH groups available for reaction with NEM. The GSH pool was completely extinguished while the proteomic pool was decreased by approximately 50 nmol (115 nmoles of SH vs 166 nmol in proteome without GSH). As a result, the amount of proteomic Zn^{2+} that became available for reaction with ZQ diminished.

Reaction of the Zn-proteome with TSQ and NEM

When the experiment was repeated using TSQ (Figure 8), the results were quite different. The reaction of the Zn-proteome with TSQ yielded fluorescence confined only to the proteomic fractions (Figure 8A). These fractions had a characteristic blue-shifted emission spectrum as seen with TSQ binding to Zn-proteins to form adducts (λ_{MAX} , 470 nm).¹⁵ Also, unlike Zinquin, TSQ was unable to remove any Zn^{2+} from the modified Zn-proteome to form $\text{Zn}(\text{TSQ})_2$ (Figure 8B). Strikingly, the reaction NEM with this TSQ-Zn-Proteome was vastly different in comparison with the reaction of NEM with the ZQ-Zn-Proteome. Instead of releasing Zn^{2+} to the LMW pool as in the case with Zinquin, all of the fluorescence remained within the proteomic region. The emission spectrum was still centered about 472 nm; however the intensity increased nearly 90% vs. control. Apparently, TSQ-Zn-protein

adducts comprised the fluorescent product. Moreover, new Zn^{2+} binding sites that had been altered by reaction with NEM became available to react with TSQ, resulting in a large, overall increase in fluorescence intensity.

Subcellular Distribution of Zinquin Fluorescence

Reports on the distribution of cellular Zn^{2+} have suggested that the sources of Zn^{2+} being sensed by Zinquin and TSQ are confined within intracellular vesicles, sometimes referred as “zincosomes.”^{19, 26} One study suggested the accumulation of labile Zn^{2+} within these vesicles after exposure to NEM.¹⁹ To determine whether ZQ-Zn-proteins were associated with specific organelles, centrifugal fractionation of homogenized Zinquin-exposed C6 cells was performed in tandem with cells additionally exposed to NEM. (Figure 9) The first fractionation—considered the nuclear and cell membrane/debris fraction—accounted for $37 \pm 3\%$ (average $\pm$ standard deviation, $n=4$) of the total fluorescent intensity. The most intense fraction was that of the cytosol supernatant, which contained $53 \pm 9\%$ of the fluorescence. There did not appear to be any significant fluorescence associated with the microsomal fraction, signifying that microsomes are not a key player in the intracellular milieu of Zn^{2+} targets for Zinquin within this system. In comparing these cells with ones additionally treated with NEM, there were increases in fluorescence intensities of both the nuclear and cytosolic fractions. Comparing the emission profiles, the cytosolic fluorescence was red shifted to 483 nm after exposure to NEM, indicative of an increase in $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$, while the membrane-nuclear fractions remained near 470 nm. These data suggested that the release of free Zn^{2+} signified by the increase in the LMW $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ pool in the gel filtration experiments likely occurred within the cytosol and was not redistributed into other compartments.

Discussion

Intracellular probes for metal ions such as Zn^{2+} offer the possibility of imaging metal ion distribution and their time-resolved trafficking in live cells. In typical experiments, sensors are mixed with cells and a fluorescence output is observed microscopically using a broad-band wavelength detector. Without subsequently lysing the cells and undertaking a molecular-level analysis of the products that have been generated, it is difficult to determine what these sensors are imaging.²⁷

Commonly, it has been hypothesized that sensors for Zn^{2+} measure “free, loosely bound, labile, or mobile” Zn^{2+} that exists in reaction 1. Thus, for example, the punctuate fluorescence emission seen microscopically in cells treated with ZQ or TSQ has been thought to represent membrane enclosed vesicles of Zn^{2+} , called zincosomes.^{19, 26, 28} But even this simplest of hypotheses recognizes that the amount of Zn^{2+} that associates with the sensor depends on the relative equilibrium constants and concentrations of the probe and the Zn^{2+} binding sites represented in reaction 2. Any such interpretation must also account for the individual diffusional characteristics of the probe within the heterogeneous environment of the cell.

In this context, we reexamined the cellular reaction of NEM with sulfhydryl binding sites that results in the elevation of intracellular Zn^{2+} as observed by Zinquin and FluoZin-3.^{19–21} One of these reports also showed that NEM mobilizes ZQ detectable Zn^{2+} such that it becomes available for rapid efflux.¹⁹ In investigating these experiments, we followed the reaction of NEM with ZQ_{EE} treated C6 cells with spectrofluorimetry. The initial fluorescence emission spectrum obtained with cells incubated with ZQ_{EE} centered at 468 nm, consistent with the operation of reaction 4 and the formation of ZQ-Zn-protein adducts (Figure 3). Subsequent lysis of these cells and gel filtration chromatography to separate proteome from low molecular weight molecules showed that almost all of the fluorescence

and Zn^{2+} were confined to the proteome fractions, although a small peak of fluorescence and Zn^{2+} existed in the LMW region consistent with the presence of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ (Figure 4B).

Reaction of NEM with ZQ_{EE} exposed cells resulted in an increase in fluorescence, a broadening of the spectrum, and a red-shift of its wavelength maximum (Figure 4A). Apparently, the reaction of NEM with binding sites for Zn^{2+} in the proteome made available new sites for interaction with ZQ. The shift of the spectrum toward 490 nm was consistent with the appearance of additional $\text{Zn}(\text{ZQ})_2$.¹⁶ Gel filtration of the supernatant from these cells revealed fluorescence ($\lambda_{\text{MAX}} = 465 \text{ nm}$) in the proteome band that represented ZQ-Zn-proteins (Figure 4B). In addition, a significant low molecular weight peak of fluorescence ($\lambda_{\text{MAX}} = 492 \text{ nm}$) and Zn^{2+} signaled the formation of $\text{Zn}(\text{ZQ})_2$. The sulfhydryl content of both proteome and LMW fractions were substantially depressed, indicative of the reaction of NEM with thiolate groups (Figure 4C).¹⁷ Based upon the results, reactive thiols included ligating groups in Zn-proteins that upon reaction with NEM were no longer able to bind Zn^{2+} , thereby, either releasing Zn^{2+} or opening these sites to reaction with ZQ (reaction 5).

In this experiment, Zn^{2+} was redistributed to some extent between proteome and LMW fractions. But the total concentration of Zn^{2+} remained constant. A similar result was obtained when cells were incubated with NEM in the absence of ZQ. In contrast to a previous report, there was no indication that Zn^{2+} underwent efflux into the external medium.¹⁹

These reactions were also carried out with cell supernatant, containing Zn-proteome and LMW metal binding ligands, primarily GSH, and isolated Zn-proteome.²⁵ Results with cells and supernatant were qualitatively similar. The reaction of NEM with Zn-proteome alone was exaggerated in that much of the control proteomic Zn^{2+} reacted with ZQ_{ACID} , forming $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ that migrated as a LMW species and was identified by its characteristic fluorescence spectrum ($\lambda_{\text{MAX}} = 492 \text{ nm}$) (Figure 7). Clearly, the presence of the major pool of GSH sulfhydryl groups in the cells and supernatant diverted much of the NEM from reaction with proteomic SH groups. The results of the experiments with ZQ were consistent with reaction 7 or 8. NEM either stimulated full dissociation of Zn^{2+} from binding sites (reaction 7) rendering it fully available for reaction with ZQ (reaction 1)

or by reducing the number of ligands chelating Zn^{2+} (reaction 8) so that ligand substitution with ZQ became more appealing (reaction 4):

A study substituting TSQ for ZQ clarified the situation. As with ZQ, experiments in cells showed that TSQ formed TSQ-Zn-protein adducts (Figure 5). Upon further reaction with NEM, fluorescence intensity markedly increased. But there was no spectral red-shift, suggestive of the formation of $\text{Zn}(\text{TSQ})_2$. Thus, it appeared that the concentration of TSQ-Zn-protein complexes increased. In confirmation, gel filtration of the lysed cells did not reveal any LMW $\text{Zn}(\text{TSQ})_2$ (Figure 6B). A more dramatic contrast between ZQ and TSQ was seen in the reactions of NEM with Zn-proteome in the presence of these sensors. Whereas, much of the proteomic Zn^{2+} became associated with ZQ as $\text{Zn}(\text{ZQ})_2$, all of the proteomic Zn^{2+} was retained in the presence of TSQ as TSQ-Zn-protein ($\lambda_{\text{MAX}} = 470 \text{ nm}$) (Figure 8). The only evidence that any reaction had occurred involving Zn^{2+} was the 2 fold increase in fluorescence intensity. Evidently, reaction 6 was preferred.

We ascribe the difference in behavior of ZQ and TSQ to their distinct stability constants for binding Zn^{2+} .^{10, 29} ZQ has a substantially higher affinity for Zn^{2+} than does TSQ and, thus, competes more effectively for Zn^{2+} with the product of reaction 8. If correct, then the cellular reactions of Zn-proteome with NEM do not render Zn^{2+} fully dissociated from its binding sites. Instead, depending upon the properties of the sensor, it either remains linked to its protein binding sites through adduct formation with TSQ and to a lesser extent with ZQ_{ACID} or it undergoes full ligand substitution to form $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$. Extending these conclusions to imaging the NEM reaction with FluoZin-3, it appears that this sensor visualizes a very small concentration of basal cellular Zn^{2+} , in the pM to nM range.¹¹ Upon exposure to NEM, a 30–40 fold increase in fluorescence was reported, consistent with the chelation of Zn^{2+} as in reaction 5 in which ligand substitution leads to the transfer of Zn^{2+} from NEM-S-protein-Zn to FluoZin-3.^{20–21}

The importance of this conclusion is that it shows that fluorescent probes are not passive observers of Zn^{2+} ; they interact actively with the control and modified Zn-proteome and do so in peculiar ways depending on the properties of the sensor. Even closely related sensors such as ZQ and TSQ can react distinctly differently with intracellular sources of Zn^{2+} . Thus, chemical understanding of microscopic images of cells exposed to sensors requires complementary studies that are likely to involve extensive analysis of the internal impact of these probes on molecular Zn^{2+} distribution.

There is a further complication in rationalizing our results. In experiments to be reported elsewhere, we show that $\text{Zn}(\text{ZQ})_2$ and $\text{Zn}(\text{TSQ})_2$ can themselves react with the proteome to form sensor-Zn-protein, ternary adducts that cannot be distinguished spectrofluorimetrically from those generated by reaction of sensor with Zn-proteome (unpublished information). The detection of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ in cells exposed to ZQ_{EE} and NEM explicitly raises the question whether some of the ZQ_{ACID} -Zn-protein adduct concentration that is observed spectrofluorimetrically resulted from the reaction of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ with adventitious Zn^{2+} binding sites within the proteome:

Similarly, with TSQ this secondary reorganization might occur, though no direct evidence supported such a reaction. If this hypothetical series of reactions is correct, these sensors trigger a subtle, complex reorganization of Zn^{2+} within the proteome.

The findings presented here begin to clarify the nature of the reactions of NEM with C6 cells and probably with many other types of cells as well. NEM gains entrance to C6 cells and reacts widely with sulfhydryl groups in the proteome and with glutathione. In control cells, within detection limits, Zn^{2+} is confined to the proteome. Thus, the enhanced reaction of TSQ and ZQ with proteomic Zn^{2+} implies that NEM reacts with a significant concentration of Zn-proteins. The simplest hypothesis is that at least one of the Zn^{2+} binding ligands in these proteins is a cysteinyl sulfhydryl group. Upon reaction with NEM, the protein ligand coordination of Zn^{2+} is reduced, facilitating ligand addition or substitution by TSQ or ZQ. Nevertheless, in the absence of these sensors, Zn^{2+} remains bound within the proteome.

It has been tempting to ascribe the effects of exogenous electrophiles that target SH groups to specific reaction with metallothionein (MT), the sulfhydryl rich Zn-binding protein.³⁰ However, C6 cells contain little Zn-MT that could be detected after gel filtration chromatography (Figure 4B). Instead, the large reduction in proteomic SH groups accompanying NEM reaction with cellular thiols suggests that labilization of Zn^{2+} is widespread within the proteome. A similar picture has emerged about the reaction of NO

with cells. Substantial reductions in cellular thiols follow exposure to DEA-NO, both in proteomic and GSH pools. However, among proteins, Zn-MT is not particularly reactive.¹³ Nevertheless, as with NEM exposure, fluorescent probes detect labilized Zn²⁺.^{8, 31–32} It is likely that reactive Zn²⁺ stems from a variety of protein sources. Likewise, the actual pools of Zn²⁺ being detected are probably sensor-specific.

Finally, the *in vitro* experiments in which Zn-proteome was reacted with NEM showed that a substantial amount of Zn²⁺ in the Zn-proteome could be labilized by NEM. These reactive proteins included most of those that underwent ligand substitution with ZQ_{ACID}, consistent with the hypothesis that most of the Zn²⁺ binding sites for ZQ-Zn-proteins contain thiolate ligands. Well known examples include Zn-metallothionein and Zn-finger proteins.^{33–34} An inference is that these Zn²⁺ binding sites are vulnerable to deleterious reaction with a variety of reagents, electrophiles and oxidants, that target sulfhydryl groups.³⁰

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Abbreviations

LMW	low molecular weight
GSH	glutathione
MTT	3(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium, bromide
NEM	N-ethylmaleimide
P	protein binding sites for Zn ²⁺
SH	sulfhydryl group
TSQ	6-methoxy-8-p-toluenesulfonamido-quinoline
Zn-MT	Zinc-metallothionein
ZQ	Zinquin acid or ethylester
ZQ_{ACID}	(2-methyl-8-p-toluenesulfonamido-6-quinolyloxy)acetate)
ZQ_{EE}	ethyl(2-methyl-8-p-toluenesulfonamido-6-quinolyloxy)acetate)

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Figure 1.

A) Chemical structures of Zinquin ethyl ester, Zinquin acid, and TSQ. B) Hypothetical reaction scheme of an intracellular thiol with *N*-ethylmaleimide.

Figure 2. Toxicity of *N*-ethylmaleimide to C6 cells. Using the MTT Assay, the viability of C6 cells was determined after varying concentrations and exposure times to NEM. (Average $\pm$ standard deviation, $n = 3$)

Figure 3. Fluorescence spectra of 5×10^6 C6 cells/mL exposed to Zinquin for 30 minutes (blue) followed by treatment with $100 \mu\text{M}$ NEM for 20 minutes. Inset: Time course of ratio of fluorescence at 492 nm ('free' $\text{Zn}(\text{ZQ})_2$ species) to 467 nm (ZQ-Zn-Protein species). The gradual increase is indicative of the release of Zn^{2+} ions.

Figure 4.

Sephadex G-75 gel filtration of Zinquin-exposed C6 cells subjected with NEM. 2×10^8 C6 cells were treated with 25 μM Zinquin for 30 minutes (○) following treatment with 100 μM NEM (■). Lysed cells were fractionated via Sephadex G-75 gel filtration and fractions were analyzed for fluorescence (A), Zn^{2+} concentration (B), and thiol concentration (C). Note: B contains control cells (◇) in which unexposed cells were chromatographed.

Figure 5.

Fluorescence spectra of 5×10^6 C6 cells/mL exposed to TSQ for 30 minutes (blue) followed by treatment with $100 \mu\text{M}$ NEM for 20 minutes. Inset: Time course of ratio of fluorescence at 492 nm ('free' $\text{Zn}(\text{TSQ})_2$ species) to 475 nm (TSQ-Zn-Protein species). The invariable ratio suggests Zn^{2+} is not being released.

Figure 6.

Sephadex G-75 gel filtration of TSQ-exposed C6 cells after additional exposure to NEM. 2×10^8 cells were treated with 25 μM TSQ for 30 minutes (○) before additional exposure to 100 μM NEM. Cells were lysed the contents were separated using Sephadex G-75 gel filtration and fractions were analyzed for fluorescence (A), Zn^{2+} concentration (B), and thiol concentration (C).

Figure 7.

Reactions of the isolated Zn-proteome with Zinquin and NEM. (A) 10 μM isolated C6 proteome (based on $[Zn^{2+}]$) was reacted with 20 μM ZQ_{ACID} for 30 minutes followed by a separation using Sephadex G-50 gel filtration and analyzed for fluorescence and $[Zn^{2+}]$. (B) 10 μM isolated C6 proteome (based on $[Zn^{2+}]$) was reacted with 20 μM ZQ_{ACID} for 30 minutes followed by treatment with 100 μM NEM. The reaction mixture was separated using Sephadex G-50 gel filtration and fractions were analyzed for fluorescence and $[Zn^{2+}]$. (C) 10 μM isolated C6 proteome (based on $[Zn^{2+}]$) was supplemented with 50 nmoles of glutathione before subsequent reactions with 20 μM ZQ_{ACID} and 100 μM NEM followed by separation via Sephadex G-50 gel filtration.

Figure 8.

Reactions of the isolated Zn-proteome with TSQ and NEM. (A) 10 μM isolated C6 proteome (based on $[Zn^{2+}]$) was reacted with 20 μM TSQ for 30 minutes followed by separation using Sephadex G-50 gel filtration and analyzed for fluorescence and $[Zn^{2+}]$. (B) 10 μM isolated C6 proteome (based on $[Zn^{2+}]$) was reacted with 20 μM TSQ for 30 minutes followed by treatment with 100 μM NEM. The reaction mixture was separated using Sephadex G-50 gel filtration and fractions were analyzed for fluorescence and $[Zn^{2+}]$.

Figure 9. Subcellular distribution of fluorescence of Zinquin-exposed C6 cells treated with NEM. 2×10^8 C6 cells were exposed to $25 \mu\text{M}$ ZQ_{EE} for 30 minutes (blue) and subjected to $100 \mu\text{M}$ NEM for 30 minutes (red). Homogenized cells were differentially centrifuged to isolate nuclear, mitochondrial, lysosomal, microsomal, and cytosolic fractions. Fluorescence was recorded for each subcellular fraction. (bars indicate average intensity $\pm$ standard deviation, $n=4$)