Published in final edited form as:

Inorg Chem. 2012 March 19; 51(6): 3625–3632. doi:10.1021/ic2025236.

Reaction of Metal Binding Ligands with the Zinc Proteome: Zinc Sensors and TPEN

Jeffrey W. Meeusen^{1,2}, Andrew Nowakowski¹, and David H. Petering^{1,*}

¹Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, Milwaukee, WI 53201

Abstract

The commonly used Zn^{2+} sensors, TSQ and Zinquin, have been shown to image Zn-proteins as a result of the formation for sensor-Zn-protein ternary adducts not $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{Zinquin})_2$ complexes. The powerful, cell permeant chelating agent TPEN is also used in conjunction with these and other Zn^{2+} sensors to validate that the observed fluorescence enhancement seen with the sensors depends on intracellular interaction with Zn^{2+} . We demonstrated that the kinetics of reaction of TPEN with cells pretreated with TSQ or Zinquin was not consistent with its reaction with $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{Zinquin})_2$. Instead, TPEN and other chelating agents extract between 25–35% of the Zn^{2+} bound to the proteome, including Zn^{2+} from Zn-metlothionein, and, thereby, quench some but not all of the sensor-Zn-protein fluorescence. Another mechanism in which TPEN exchanges with TSQ or Zinquin to form TPEN-Zn-protein adducts found support in the reactions of TPEN with Zinquin-Zn-alcohol dehydrogenase. TPEN also removed one of the two Zn^{2+} ions per monomer from Zn-alcohol dehydrogenase and Zn-alkaline phosphatase, consistent with its ligand substitution reactivity with the Zn-proteome.

Introduction

Zinc is an essential trace metal thought to be a co-factor in an estimated 2800 mammalian proteins.^{1,2} In addition to its well described catalytic and structural roles, transient Zn^{2+} trafficking among protein binding sites may contribute to changes in cellular state.³ For example, as much as 10% of the zinc found in the brain is loosely bound and localized to the hippocampus, an area of the brain thought to involve memory.^{4,5} This transient pool of zinc is thought to be a chemical messenger in neural synapses.

Nutrient zinc insufficiency and deficiency readily occur in human populations.^{6,7} In experimental animals, they cause a host of adverse physiological effects that presumably result from perturbations of intracellular Zn^{2+} distribution.^{8–11} The molecular origin of such effects remains poorly understood.^{12,13}

TPEN, 2,2',2'',2'''-tetrapyridyl-ethylene diamine, is a high affinity metal ion chelator that in its deprotonated form is neutral and readily crosses cell membranes.^{14,15} Its complement of pyridyl and amine nitrogen ligands bind Zn^{2+} with an apparent log stability constant of 15.6 M^{-1} at pH 7.2.¹⁶ These properties support its use in two primary types of experiments with cells in culture. In one, TPEN is added to cells to chelate labile or loosely bound, Zn^{2+} involved in trafficking, in order to render them functionally Zn^{2+} deficient for the purpose of understanding the responses of cells to nutrient Zn^{2+} deficiency.^{17–19}

*Corresponding author. Tel.: 1 414 229 5853; fax: 1 414 229 5530. petering@uwm.edu (D.H. Petering)..

²Present Address: Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN 55905

In the other, after incubation with a Zn-fluorescent sensor, TPEN is added to cells, undergoes reaction 2, and, thereby, validates that the fluorescence observed is due to the actual formation of Zn-sensor complexes:^{14,20}

The current experiments revisit these types of experiments for two interrelated reasons. First, TPEN is an exceptionally strong, promiscuous metal ion chelator.^{16–19} Instead of binding only free or loosely bound Zn^{2+} that enters the cell, mimicking nutrient Zn^{2+} deficiency, it might undergo ligand substitution with Zn-proteins and, likewise, disturb the normal distribution of other metals such as Fe and Cu:

Second, it has been demonstrated recently that the commonly used Zn^{2+} sensors TSQ and Zinquin react primarily with Zn-proteins to form TSQ-Zn-protein ternary complexes not with intracellular Zn^{2+} to produce $\text{Zn}(\text{TSQ})_2$.^{21,22} TPEN quenches much of the fluorescence emission that results the reaction of these Zn^{2+} sensors with cells. Previously, it was assumed that the reaction of TPEN with $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{ZQ})_2$ accounted for the observed fluorescence quenching. With the new understanding of the mechanism of fluorescence enhancement in cells exposed to these sensors, the underlying chemistry of TPEN dependent quenching reaction needs to be reconsidered in an investigation of possible reactions of TPEN with sensor-Zn-proteins. In one hypothetical reaction, TPEN competes for Zn^{2+} bound in the ternary complex or competes with TSQ or ZQ for binding to Zn-proteins:

In the other, TPEN exchanges with the sensor in the ternary adduct:

The impetus to undertake this study stems in part from the lack of recognition that TPEN with its powerful metal ion chelating properties might well react with Zn-proteins as well as loosely bound Zn^{2+} .^{14,16–20,23–26} The present experiments provide support for hypotheses that reactions 3–5 operate in cells.

Methods

Cell culture

LLC-PK₁ cells were purchased from the American Tissue Culture Company. Medium 199 with HEPES growth media was supplemented with 50,000 units/L Penicillin G, 50 mg/L streptomycin and 4% fetal calf serum. Flasks were incubated under 5% CO₂ at 37° C and the medium was changed every 48 hours. Cultures were subdivided by trypsin/EDTA treatment every 5–7 days.

Reactions and spectrofluorimetry of cells or cell lysates exposed to TSQ or ZQ followed by TPEN

Exposures to TPEN, ZQ, TSQ, pyrrithione, or ZnCl_2 were conducted at concentrations that did not affect viability as measured by the MTT assay.²⁷ Cells were grown in 35 mm culture plates and rinsed three times with Dulbecco's phosphate buffered saline (DPBS) prior to

being stained for with TSQ or ZQ. After staining, cells were again rinsed three times and finally suspended in DPBS. Lysates were prepared from TSQ/ZQ stained cells by sonicating cells in 50 mM TRIS-Cl, pH 7.4, and clarifying by centrifugation at 47,000 or 100,000 g. Cell suspensions or supernatants in the above buffer were added to a fluorescence cuvette. Their emission spectra were recorded between 400–600 nm with a Hitachi F4500 spectrofluorometer using an excitation wavelength of 360 nm and a temperature of 25° C. All experiments with cells or supernatants were conducted under these conditions.

Sephadex G-75 chromatography and isolation of Zn-proteome, and metal analysis

Sephadex G-75 size exclusion chromatography beads were purchased from Sigma. Beads were initially hydrated in 20 mM Tris-Cl pH 7.4. Columns were 0.75 mm × 90 cm, sample was eluted by gravity reservoir, and 20 drop fractions were collected. The void volume was ~8mL and fractions 10–20 contained molecules larger than 10 kDa, referred to as the proteome. A subset of these proteins would be the Zn-proteome. Fractions 20–30 included molecules ~10 kDa in size, and fractions 30–40 were comprised of the low molecular weight species. The 10 kDa species Zn-containing species was identified as MT by amino acid analysis.²¹

The metal content of each fraction was measured by flame atomic absorption spectrophotometry (AAS). The GBC model 904AA instrument atomized samples with an acetylene torch using an 80:20 mix of compressed air and acetylene. Measurements were obtained with a Zn element lamp and a deuterium background lamp. Data acquisition was performed in running mean mode and calibrated prior to each run using standards of 7.7, 15.4, and 30.8 μM Zn^{2+} that correspond to 0.5, 1.0, and 2.0 $\mu\text{g/mL}$ Zn^{2+} .

Reactions of TPEN with model Zn-proteins

Bovine erythrocyte alkaline phosphatase (AP) was obtained from Sigma. This protein, which binds 2 Zn^{2+} per monomer was prepared to a concentration of 25 mg/mL by dissolving in degassed 50 mM HEPES and 0.1 M KNO_3 buffer, pH 7.2. Yeast alcohol dehydrogenase was purchased from Worthington Biochemical Corporation. It contains 2 Zn^{2+} per monomer. The protein was prepared by dissolving the lyophilized powder in degassed 20 mM Tris-Cl buffer, pH 7.4 to a final concentration of 10 mg/mL. Fractions were assayed for Zn^{2+} by AAS and protein by the Lowry Assay (BIO-Rad DC Protein Kit).²⁸ Proteins were mixed with TSQ or ZQ at indicated concentrations prior to addition of TPEN in the above buffers at 25° C.

Activity Assays for Zn_2 -ADH and Zn_2 -AP

Zn_2 -ADH or Zn-ADH (0.05–01 μM) was reacted with 0, 2.5, 5, 10, 25, 50, 75 and 100 mM ethanol in the presence of 15 mM NAD in 10 mM $\text{Na}_2\text{P}_2\text{O}_7$ buffer pH 8.8 at 25° C. The conversion of NAD to NADH was monitored at 340 nm for 30 seconds and an initial rate was measured as absorbance/min. Zn_2 -AP or Zn-AP (0.075 μM) was reacted with 0, 10, 25, 50, 100, 250, 500, 1000, and 2000 μM 4-nitrophenyl phosphate in 50 mM HEPES buffer pH 8.2. The conversion of 4-nitrophenyl phosphate to 4-nitrophenol was monitored at 405 nm for 30 seconds and an initial rate was determined as absorbance/min.

Kinetic analysis of reactions

Formation of Zn-TPEN upon ligand substitution reaction of pseudo-first order excesses of TPEN with $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{ZQ})_2$ was monitored by loss of fluorescence or change in absorbance, respectively. A plot of $\ln[\text{Zn}(\text{sensor})_2]$ vs. time in seconds yielded a linear curve. The slope of the line was the best-fit linear regression and was called k_{OBSERVED} .

Results

Toxicity of TPEN and Zn-TPEN

LLC-PK₁ cells were completely viable after exposure to 100 μ M TPEN for one hour. However, 24 hour exposure reduced cell viability to 30% (Figure 1). The effect was ameliorated by addition of equimolar ZnCl₂ to the TPEN reagent. The increased survival of cells exposed to Zn-TPEN compared to cells exposed to TPEN alone, suggested that at least some of the toxicity of TPEN was derived from its metal chelating properties.

TPEN quenching of TSQ dependent cellular fluorescence-comparison with Zn(TSQ)₂

LLC-PK₁ cells stained with TSQ displayed a fluorescence λ_{max} at 470 nm, indicative of the formation of TSQ-Zn-protein adducts.²¹ Subsequent incubation of a cell suspension containing 5 μ M Zn²⁺ with 100 μ M TPEN resulted in a 90% loss of fluorescence signal in seven minutes that followed first order kinetics with a k_{obs} of $2.7 \times 10^{-3} \text{ s}^{-1}$ (Figure 2A). The λ_{max} of the residual fluorescence in the TPEN treated cells remained at 470 nm.

In a related experiment, cell supernatant containing 5 μ M Zn²⁺ was mixed with 10 μ M TSQ. The resulting fluorescence emission spectrum exhibited a λ_{max} at 470 nm, consistent with the formation of TSQ-Zn-proteins. Addition of 100 μ M TPEN resulted in a first order decay in fluorescence with a k_{obs} of $2.1 \times 10^{-3} \text{ s}^{-1}$ (Figure 2A). Using 100 μ M TPEN, cells and supernatant containing the same concentration of total Zn²⁺ and pretreated with TSQ reacted with similar pseudo-first order kinetics.

For comparison, we examined the reaction of 100 μ M TPEN with 5 μ M Zn(TSQ)₂, the Zn²⁺ species that had previously been thought to account for TSQ dependent cellular fluorescence. This reaction reached 90% completion in less than 100 seconds and was characterized by a pseudo-first order rate constant of $2.5 \times 10^{-2} \text{ s}^{-1}$ (Figure 2A). According to these results, the induced loss of fluorescence caused by TPEN was fivefold faster with Zn(TSQ)₂ than with TSQ stained cells or supernatant exposed to TSQ. Each of these reactions was carried out under pseudo-first order conditions for TPEN (100 μ M) and the same concentration of Zn²⁺. Because TSQ-incubated cells and isolated cell supernatant reacted at similar rates with TPEN, it did not appear that diffusion of TPEN into cells was a rate-limiting event in this process. Thus, it was concluded that reaction of TPEN with intracellular Zn(TSQ)₂ did not account for the cellular reaction that caused quenching of cellular fluorescence.

Characterizing the reaction of TPEN with Zn(TSQ)₂ further, a series of pseudo-first order excess concentrations of TPEN was reacted with 2.5 μ M Zn(TSQ)₂ and the formation of Zn-TPEN monitored by the loss of fluorescence. Plotting the k_{OBSERVED} for the rate of Zn(TSQ)₂ decay against the concentration of TPEN revealed a linear relationship with a slope of $99 \text{ s}^{-1} \text{ M}^{-1}$ representing the second order rate constant (k_2) for part of the reaction and a non-zero intercept of $2.7 \times 10^{-3} \text{ s}^{-1}$ (Figure 2B). The second order part of the rate equation is taken to represent the direct bimolecular reaction of TPEN with Zn(TSQ)₂. The first order, TPEN-independent process, is hypothesized to involve rate limiting dissociation of a bulky quinolinesulfonamide moiety from the Zn²⁺ ion prior to rapid reaction of the remaining Zn-TSQ complex with TPEN.

Quenching of TSQ dependent cellular fluorescence by TPEN in the presence of extra Zn²⁺

Another common tool in experiments about Zn²⁺ trafficking is the use of the ionophore pyrrhione (PYR), which forms hydrophobic Zn(PYR)₂ complexes, to shuttle Zn²⁺ rapidly into cells. Incubation of 10⁶ cells for 10 min with 3 μ M PYR in the presence of 30 μ M ZnCl₂ resulted in a large increase in the fluorescence signal of intracellular TSQ. The signal

increase was accompanied by a shift in λ_{max} from 470 nm to 490 nm, consistent with the formation of $\text{Zn}(\text{TSQ})_2$ through the reaction of TSQ with $\text{Zn}(\text{PYP})_2$ (Figure 3).²¹ Addition of 100 μM TPEN reduced the fluorescence signal by nearly half within the time of mixing and then by about 50% again within two minutes. During this period, the λ_{max} of the remaining signal moved toward 470 nm. Evidently, the rapid reaction represented the interaction of TPEN with $\text{Zn}(\text{TSQ})_2$. The remaining fluorescence (77%) declined slower. The initial, swift reaction involved TPEN and intracellular $\text{Zn}(\text{TSQ})_2$. Its rapidity verified that the flux of TPEN into the cells was, itself, a quick process that was not significantly rate limiting for the overall reaction. The slower decay process resulted from the quenching of the fluorescence of the original TSQ-Zn-protein adducts. This experiment, in particular, emphasized that the native fluorescent species involving TSQ is not $\text{Zn}(\text{TSQ})_2$, as neither its kinetics of reaction with TPEN nor its fluorescence emission spectrum matched results of the reaction of TPEN with native cells or cell supernatant.

TPEN quenching of Zn^{2+} dependent ZQ fluorescence in LLC-PK₁ cells and Zn-proteome

Zinquin is a Zn^{2+} sensor closely related to TSQ.²² To investigate the generality of the results with TSQ, the capacity of TPEN to quench the fluorescence of ZQ was examined in LLC-PK₁ cells (approximately 5 μM total Zn^{2+}) pretreated with 10 μM ZQ_{EE} , the esterified sensor that rapidly accumulates in cells. Upon cellular uptake, ZQ_{EE} was assumed to hydrolyze to ZQ_{ACID} , which, in turn, reacted to generate a Zn^{2+} -based fluorescence emission spectrum centered at 470 nm. As with TSQ, this spectrum is characteristic of the presence of ZQ-Zn-protein adduct species.²² All of the fluorescence was associated with the proteome fraction consistent with the production of ZQ_{ACID} -Zn-protein adducts.²² When these cells were exposed to TPEN, a multi-phasic reaction took place in which about 70% of the fluorescence was quenched in the major first order reaction (Figure 4A). This result was compared to the reactions of 5 μM of isolated Zn-proteome with 10 μM ZQ_{EE} or ZQ_{ACID} followed by quenching with 100 μM TPEN. The quenching of fluorescence of the ZQ_{ACID} -exposed proteome with TPEN occurred with a qualitatively similar kinetic profile as seen in the intact cells, although the major step involving about 70% of the fluorescence occurred somewhat faster than in cells. ZQ_{EE} reacted more slowly. These results supported the hypothesis that TPEN was rapidly accumulated in cells and then reacted with the ZQ_{ACID} -Zn-proteome as seen *in vitro*.

Reaction of TPEN with, $\text{Zn}(\text{ZQ}_{\text{EE}})_2$, and $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$

As models for the intracellular reactions of TPEN with ZQ-based complexes, the reactivity of TPEN with $\text{Zn}(\text{ZQ}_{\text{EE}})_2$ and $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ was determined. Like $\text{Zn}(\text{TSQ})_2$, $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ underwent rapid reaction with TPEN (Figure 4B). However, this reaction was zero order in TPEN and was characterized by a first order rate constant of $4.8 \times 10^{-2} \text{ s}^{-1}$. Unexpectedly, the reaction of $\text{Zn}(\text{ZQ}_{\text{EE}})_2$ with TPEN proceeded at a much slower rate (Figure 4B). Moreover, it occurred with second order kinetics, first order in both $\text{Zn}(\text{ZQ}_{\text{EE}})_2$ and TPEN ($k = 1.82 \text{ M}^{-1} \text{ s}^{-1}$) with a non-zero y-intercept of $3.0 \times 10^{-4} \text{ s}^{-1}$.

The pseudo-first order rates of reaction of TPEN with $\text{Zn}(\text{TSQ})_2$ and $\text{Zn}(\text{ZQ})_2$ species were either much faster or slower than the observed reactions in cells, consistent with the idea that ZQ-Zn-protein species not Zn^{2+} -sensor complexes are predominantly present in cells and undergo reaction with TPEN. Moreover, the relatively fast reaction of TPEN with ZQ_{EE} -treated cells in comparison with its reaction with ZQ_{EE} -Zn-proteome supported the conclusion that ZQ_{EE} was rapidly hydrolyzed to ZQ_{ACID} once in cells. Finally, the difference in kinetic behavior of $\text{Zn}(\text{ZQ})_2$ species with TPEN was attributed to a much faster dissociation rate of ZQ_{ACID} from $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ than ZQ_{EE} from $\text{Zn}(\text{ZQ}_{\text{EE}})_2$, due to the electrostatic repulsion between the two negatively charged ZQ_{ACID} ligands.

Reaction of TPEN with cellular proteomic Zn²⁺

Under standard culture conditions, LLC-PK₁ cells contained 16 ± 4.2 nmol Zn²⁺/10⁷ cells. Incubation of these cells with 25 μ M TPEN for 30 minutes prior to isolation of cell supernatant did not significantly alter the total Zn²⁺ recovered (Table 1). Quantifying the distribution of supernatant Zn²⁺ after Sephadex G-75 chromatography revealed that the proteomic Zn²⁺ band retained 90–100% of its total Zn²⁺. The MT fraction was reduced by 60% while the low-molecular weight Zn²⁺ increased by 40%, indicative of the formation of Zn-TPEN. Increasing the concentration of TPEN to 100 μ M, a concentration commonly employed in cellular experiments, reduced proteomic Zn²⁺ by 30 %.^{29,30} This reduction was accompanied by a 225 % increase in low molecular weight Zn²⁺ thought to be Zn-TPEN. The small amount of Zn₇-MT (3.8 nmol/10⁷ cells) typically present in LLC-PK₁ cells was substantially reduced.

Reaction of TPEN and other ligands with Zn-proteins

Reactions 4 and 5 summarize how TPEN might quench the fluorescence of ZQ_{ACID}-Zn-proteins. The first of these involves successful competition for proteomic Zn²⁺ by TPEN. A published experiment showed that a series of ligands differing in conditional stability constant for Zn²⁺ at pH 7 between 15.6 and 8.0 removes 20–30% of the Zn²⁺ from isolated Zn-proteome.³¹ In the present experiment, Zn-proteome from LLC-PK₁ cells was reacted with a ten-fold excess of competing ligand (100 μ M) for 30 min and the extent to ligand substitution transfer of Zn²⁺ from the proteome to the ligand determined by Sephadex G-75 chromatography. Using TPEN, EDTA, TREN, EGTA, and NTA as ligands spanning a 10⁸ range in conditional stability constant, we observed that each sequestered about 30% of the proteomic Zn²⁺, suggestive that all of them might be reacting with the same set of Zn-proteins (Table 2). The reacted proteomes were isolated again by Sephadex G-75 chromatography and then tested for reactivity with Zinquin. In each reaction, less fluorescence was observed than been detected when the native proteome was probed with Zinquin.

The extent of suppressed fluorescence was qualitatively and inversely related to the stability constant as seen in Table 2. For example, initial reaction of TPEN with the Zn-proteome resulted in 75% loss in fluorescence, whereas NTA depressed fluorescence only 35%. These findings implied that these ligands react with different subsets of the Zn-proteome that, in turn, are differentially involved in binding ZQ_{ACID}. Moreover, the results with TPEN indicated that up to 75% of the quenching of cellular ZQ fluorescence described above might be attributable to chelation of Zn²⁺ bound in ternary, ZQ-Zn-protein adducts. In addition, residual TPEN, itself, may remain bound to Zn-proteins as TPEN-containing adducts. According to this picture, the residual fluorescence that remained (25%) represented ZQ-Zn-protein adducts that were able to form because TPEN had not sequestered Zn²⁺ from these Zn-proteins. If high concentrations of TPEN were present as in cells, the residual fluorescence might also be quenched as ZQ was replaced competitively by TPEN resulting in the formation of TPEN-Zn-proteins.

TPEN reaction with ZQ-Zn-ADH and ZQ-Zn-AP

The reactions of TPEN with mixtures of ZQ and Zn₂-ADH or Zn₂-AP were investigated as models for the behavior of TPEN with ZQ-treated Zn-proteome. Previously, it was demonstrated that ZQ_{ACID} readily chelated 50% of the Zn²⁺ in each protein, assumed to represent one of the two Zn²⁺ ions in each protein monomer, as Zn(ZQ_{ACID})₂.²² The fluorophore also formed a fluorescent adduct with the other Zn²⁺ in Zn-ADH (ZQ_{ACID}-Zn-ADH) but did not react with the second site in Zn-AP.²²

The reaction of 100 μM TPEN with a mixture of 5 μM Zn_2ADH and 20 μM ZQ_{ACID} quenched the fluorescence of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ as well as $\text{ZQ}_{\text{ACID}}\text{-Zn-ADH}$, but did not extract the second Zn^{2+} ion from ADH according to Sephadex G-75 analysis of the product mixture (Figures 5A and B). Moreover, reactions of 5 μM $\text{Zn}_2\text{-Proteins}$ with 100 μM TPEN removed only one Zn^{2+} (Figure 5D and E). This implied that TPEN replaced ZQ_{ACID} in a ternary complex with $\text{Zn}_A\text{-ADH}$:

As expected, addition of TPEN to the reaction mixture of $\text{Zn}_2\text{-AP}$ and ZQ_{ACID} also destroyed the resident fluorescence from $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ but like ZQ_{ACID} , did not remove Zn_A^{2+} from $\text{Zn}_A\text{-AP}$ (Figures 5A and C). Control reactions of TPEN with $\text{Zn}_2\text{-ADH}$ and $\text{Zn}_2\text{-AP}$ revealed that TPEN was reactive with only one of the two Zn^{2+} ions in each of these proteins.

Activity assays were performed on both $\text{Zn}_A\text{-AP}$ and $\text{Zn}_A\text{-ADH}$ after chelation of Zn_B . In each case, the activities were diminished (27% for $\text{Zn}_A\text{-ADH}$ and 19% for $\text{Zn}_A\text{-AP}$) compared to those of the holoenzymes. Since the activities were only modestly diminished, the results were consistent with the hypothesis that the structural Zn^{2+} , identified as Zn_B^{2+} , not the catalytic Zn^{2+} was removed in these reactions.

These reactions displayed the range of activity of TPEN in quenching ZQ_{ACID} associated fluorescence. TPEN can sequester Zn^{2+} from protein binding sites and, thereby, erase fluorescence due to $\text{ZQ}_{\text{ACID}}\text{-Zn-protein}$ adduct formation. It may accomplish the same end by substituting for ZQ_{ACID} in such ternary adducts. It can also extract Zn^{2+} from sites that do not react with ZQ_{ACID} .

Discussion

It is commonly assumed that Zn^{2+} fluorescent sensors image Zn^{2+} that is thermodynamically and kinetically available for chelation under the prevailing conditions within the cell.^{35,36} Since there is little if any free, aquated Zn^{2+} , it is assumed that the sensor, S, competes with Zn-complexes within the cell depending on their apparent stability constants for Zn^{2+} , their concentrations, and the mechanistic opportunity for ligand substitution between them:^{37,38}

Depending on changes in intracellular chemical conditions, various Zn^{2+} binding sites may be modified to alter their reactivity with the fluorescent sensor. For example, enhanced fluorescence of TSQ and other sensors after exposure of cells to nitric oxide has been interpreted as resulting from modification of sulfhydryl ligands in Zn-proteins such as metallothionein that released Zn^{2+} for reaction with the sensor.^{29,39}

Recent studies have demonstrated that TSQ and Zinquin primarily image intracellular Zn-proteins in a variety of cell types.^{21,22} They do so by forming ternary fluorescent adducts with a subset of the Zn-proteome (e.g.):

In studying the nature of the reaction of TSQ with cells, we followed the usual protocol: cells were incubated with TSQ and enhanced fluorescence emission was recorded.²¹ Then, exogenous Zn^{2+} was added to the cells in the form of the permeant complex, $\text{Zn}(\text{pyrithione})_2$ to show that a source of Zn^{2+} would cause further enhancement in fluorescence. Finally, TPEN, a powerful, cell permeant chelating agent for Zn^{2+} , was supplied to quench the fluorescence and, thereby, confirm that the fluorescence emission was due to the formation of the $\text{Zn}(\text{TSQ})_2$ complex.¹⁶

It was assumed that all of the fluorescence enhancement following addition of $\text{Zn}(\text{pyrithione})_2$ resulted from the formation of $\text{Zn}(\text{TSQ})_2$, which readily reacts with TPEN (Figures 2 and 3). With the emergence of the robust hypothesis that TSQ actually fluoresces due to the formation of TSQ-Zn-proteins, it became necessary to reconsider how TPEN quenches the fluorescence of such adducts.²¹ Reactions 4 and 5 describe the two alternatives that were explored. TPEN may compete with TSQ-Zn-protein for Zn^{2+} , forming Zn-TPEN and depriving TSQ of its Zn-binding site. Or, TPEN might compete directly with TSQ for binding to the Zn-protein, excluding TSQ from the protein binding site for Zn^{2+} .

Initially, the first explanation was investigated, starting with a survey of the toxicity of TPEN in LLC-PK₁ cells. Upon incubation for 1 h with 100 μM TPEN, a concentration regularly used in Zn^{2+} sensor experiments, cells displayed no gross evidence of toxicity (Figure 1). However, over a period of 24 h, cells experienced a TPEN concentration dependent loss of viability. An obvious hypothesis to explain this result was that intracellular TPEN had extracted Zn^{2+} from key Zn-proteins or otherwise disrupted Zn^{2+} trafficking. Consistent with this view, substitution of Zn-TPEN for TPEN significantly ameliorated toxicity, presumably because Zn-TPEN did not have the capacity to compete for intracellular Zn^{2+} . Nevertheless, the fact that Zn-TPEN, itself, displayed some toxicity implied that reactions other than Zn-chelation might come into play when cells were exposed to this molecule.

Fluorescence quenching by TPEN in cells previously incubated with either TSQ or ZQ_{EE} occurred at slower rates than comparable reactions of TPEN with $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ observed *in vitro* and in cells with $\text{Zn}(\text{TSQ})_2$ (Figures 2 and 4). These results provided additional support for the conclusion that neither TSQ nor ZQ primarily imaged Zn^{2+} .^{21,22} Instead, if sensor-Zn-protein adducts were formed, other modes of fluorescence quenching must be involved.

TPEN is a potent Zn^{2+} chelating agent and it would not be surprising if it effectively competed with intracellular pools of Zn^{2+} other than $\text{Zn}(\text{TSQ})_2$ or $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ to form Zn-TPEN.¹⁶ Indeed, an earlier report demonstrated that multidentate ligands including TPEN that varied in log apparent stability constant for Zn^{2+} at pH 7 between 8 and 16 removed 20–30% of the total Zn^{2+} from isolated Zn-proteome.³¹ The present experiments conducted with LLC-PK₁ cells confirm that TPEN undergoes extensive ligand substitution with members of the Zn-proteome including Zn-metallothionein (reaction 4) over time (Table 1). In the process, the reactivity of the residual Zn-proteome with ZQ_{ACID} declined 75% in comparison with un-treated Zn-proteome. Thus, it is likely that TPEN quenches TSQ related fluorescence successfully by competing for protein associated Zn^{2+} which is bound to TSQ.

In the course of these experiments, we compared the properties of reaction of the Zn-proteome with TPEN and other Zn^{2+} chelators (Table 2). Under similar conditions, several

multidentate ligands extracted similar fractions of proteomic Zn^{2+} , suggestive of a common pool of Zn^{2+} that is relatively available for ligand substitution reaction. But when the residual Zn-proteome was interrogated with ZQ_{ACID} , strikingly different amounts of fluorescence enhancement were observed, ranging from 24% for EGTA to 85% for EDTA. The wide span of reactivity indicated that members of this set of ligands accessed different Zn-proteins in their reactions with the Zn-proteome. Considering the large span of Zn^{2+} stability constants and variety of structures of this group of chelating agents, these results signal that ligand substitution with proteome-bound Zn^{2+} is likely to be a common reaction when cells are treated with reagents that have even intermediate Zn^{2+} binding affinity.

TSQ and ZQ behaved differently than these chelating agents with the Zn-proteome. Neither TSQ nor ZQ_{ACID} mobilized Zn^{2+} out of the LLC-PK₁ proteome or the Zn-proteomes from a variety of other cell types.²² The overall log conditional stability constant of $\text{Zn}(\text{ZQ}_{\text{ACID}})_2$ at pH 7 is 13, indicating that ZQ_{ACID} should also compete effectively for proteomic Zn^{2+} .⁴⁰ Perhaps, there is a kinetic barrier to reaction that plays a role in its inactivity. We hypothesize that the rate limiting step in ligand substitution for the ligands we studied may be the replacement of the third or fourth metal binding group of the protein with a group from the competing ligand. For multidentate ligands with several intramolecular ligating groups, this is feasible. But with bidentate ligands, such as TSQ and ZQ_{ACID} , less favorable intermolecular addition must take place and overall ligand substitution becomes difficult. In this circumstance, adduct formation is favored.

In the course of these experiments, we also surveyed the effects of TPEN on the intracellular distribution of other transition metals. Just as this powerful ligand removed Zn^{2+} from the proteome, so too, it competed for 20% of the proteomic Fe and significant fractions of proteomic and MT-bound Cu (data not shown). These results underscore that TPEN is a promiscuous chelating agent of transition metal ions in cells. Because it competed directly with the proteome for Zn^{2+} , TPEN cannot be thought of as an innocent substitute for Zn-depleted extracellular medium in the induction of cellular Zn^{2+} deficiency, despite its frequent use for this purpose.^{17–19} The fact that Zn-saturated TPEN displayed some toxicity emphasizes that this hexadentate ligand interacts in multiple ways with cells besides simply reacting with free Zn^{2+} . This includes reaction with Fe and Cu pools and forming ternary complexes with Zn-proteins.

In vitro analysis of the reactions of TPEN with Zn_2 -ADH and Zn_2 -AP provides a model for two unique modes of TPEN perturbation of cellular Zn^{2+} . That TPEN was able to remove one of the Zn^{2+} ions from either Zn_2 -ADH or Zn_2 -AP agreed with the Zn-proteomic findings that TPEN was capable of sequestering some but not all of the proteomic Zn^{2+} (Table 2). In addition, TPEN competed successfully with ZQ_{ACID} -Zn-ADH to form what we hypothesize to be TPEN-Zn-ADH (Table 3 and Figure 6). Besides serving as a second mechanism that quenches the fluorescence that results from the formation of TSQ- or ZQ-Zn-proteins, this finding also opens the possibility that TPEN alters cell behavior, in part, by binding to Zn-proteins.

The present study demonstrates that the activity of TPEN in living cells is more complicated than commonly assumed. Not only is it expected to be reactive with *loosely bound* Zn^{2+} that might be involved in processes of Zn^{2+} trafficking; it also competes successfully for a significant percentage of proteome-bound Zn^{2+} . Moreover, it can form adduct species with Zn-proteins. Thus, it is not an innocent by-stander in cells that serves as an experimental substitute for nutrient Zn^{2+} deficiency. The fact that TPEN can participate in both reactions 4 and 5 provides an explanation for its ability to quench the fluorescence of TSQ- and ZQ-Zn-protein adducts and further strengthens the hypothesis that such ternary complexes account for much of the observed fluorescence emission in cells exposed to these sensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding was made possible by the NIH grants GM-85114 and ES-04184. Initial funds were from the University of Wisconsin – Milwaukee Research Growth Initiative. Metal analyses were conducted with the aid of the Children's Environmental Health Sciences Core Center. We thank Susan Krezoski for her technical support.

Abbreviations

AAS	atomic absorption spectrophotometry
CA	bovine serum carbonic anhydrase
DPBS	Dulbecco's phosphate buffered saline
DTNB	5,5-dithiobis-2-nitrobenzoic acid
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid
MT	metallothionein
MTT	3(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium, bromide
4-NPA	<i>p</i> -nitrophenyl acetate
NTA	nitrilotriacetate
PYR	pyrithione
sensor	TSQ or ZQ
TREN	Tris(2-aminoethyl)amine
TSQ	6-methoxy-8- <i>p</i> -toluenesulfonamido-quinoline and Figure 1
TPEN	<i>N,N,N',N'</i> -tetrakis(–)[2-pyridylmethyl]-ethylenediamine
ZQ_{ACID} Zinquin acid	(2-methyl-8- <i>p</i> -toluenesulfonamido-6-quinolyloxy)acetate)
ZQ	ZQ _{ACID} or ZQ _{EE}
ZQ_{EE} Zinquin ethylacetate	ethyl[2-methyl-8- <i>p</i> -toluenesulfonamido-6-quinolyloxy]acetate)

References

- (1). Andreini C, Banci L, Bertini I, Rosato A. J. Proteome Res. 2006; 5:196–201. [PubMed: 16396512]
- (2). Andreini C, Banci L, Bertini I, Rosato A. J. Proteome Res. 2006; 5:3173–8. [PubMed: 17081069]
- (3). Vallee BL, Auld DS. Faraday Discuss. 1992; 93:47–65. [PubMed: 1290939]
- (4). Budde T, Minta A, White JA, Kay AR. Neuroscience. 1997; 79:347–58. [PubMed: 9200720]
- (5). Lu YM, Taverna FA, Tu R, Ackerley CA, Wang YT, Roder J. Synapse. 2000; 38:187–97. [PubMed: 11018793]
- (6). Prasad AS. J Am Coll Nutr. 2009; 3:257–65. [PubMed: 20150599]
- (7). Cole CR, Lifshitz F. Pediatr Endocrinol Rev. 2008; 4:889–96. [PubMed: 18552751]
- (8). Walsh CT, Sandstead HH, Prasad AS, Newberne PM, Fraker PJ. Environ. Health Perspect. 1994; 102(Suppl 2):5–46. [PubMed: 7925188]

- (9). Fraker PJ, King LE. *Annu. Rev. Nutr.* 2004; 24:277–98. [PubMed: 15189122]
- (10). Fraker PJ. *J. Nutr.* 2005; 135:359–62. [PubMed: 15735063]
- (11). Fukada T, Kambe T. *Metallomics.* 2011; 3:662–74. [PubMed: 21566827]
- (12). Daniel H, tom Dieck H. *Biol. Chem.* 2004; 385:571–83. [PubMed: 15318805]
- (13). Ho E, Dukovcic S, Hobson B, Wong CP, Miller G, Hardin K, Traber MG, Tanguay RL. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 2011 Epub.
- (14). Zalewski PD, Forbes IJ, Betts WH. *Biochem. J.* 1993; 296:403–8. [PubMed: 8257431]
- (15). Arslan P, Di Virgilio F, Beltrame M, Tsien RY, Pozzan T. *J. Biol. Chem.* 1985; 260:2719–27. [PubMed: 3919006]
- (16). Anderegg G. *Chim. Acta.* 1977; 60:123–140.
- (17). Lu H, Pang W, Hu YD, Yang HP, Huang CY, Jiang YG. *Nutr Neurosci.* 2011; 14:80–7. [PubMed: 21605504]
- (18). Shin JH, Jung HJ, An YJ, Cho YB, Cha SS, Roe JH. *Proc. Natl. Acad. Sci. U S A.* 2011; 108:5045–50. [PubMed: 21383173]
- (19). Bernhardt ML, Kim AM, O'Halloran TV, Woodruff TK. *Biol Reprod.* 2011; 84:526–36. [PubMed: 21076080]
- (20). Tang ZL, Wasserloos KJ, Liu X, Stitt MS, Reynolds IJ, Pitt BR, St. Croix CM. *Mol. Cell. Biochem.* 2002; 234–235:211–7.
- (21). Meeusen JW, Tomasiewicz H, Nowakowski A, Petering DH. *Inorg. Chem.* 2011; 50:7563–7573. [PubMed: 21774459]
- (22). Nowakowski A, Meeusen JW, Petering DH. *Inorg. Chem.* 2011; 50:10124–10133. [PubMed: 21905645]
- (23). Lee JY, Kim JS, Byun HR, Palmiter RD, Koh JY. *Brain Res.* 2011; 1418:12–22. [PubMed: 21911210]
- (24). Kruczek C, Görg B, Keitel V, Pirev E, Kröncke KD, Schliess F, Häussinger D. *Glia.* 2009; 57:79–92. [PubMed: 18709649]
- (25). Devergnas S, Chimienti F, Naud N, Pennequin A, Coquerel Y, Chantegrel J, Favier A, Seve M. *Biochem Pharmacol.* 2004; 68:699–709. [PubMed: 15276077]
- (26). Sauer GR, Smith DM, Cahalane M, Wu LN, Wuthier RE. *J Cell Biochem.* 2003; 88:954–69. [PubMed: 12616534]
- (27). Hatok J, Babusikova E, Matakova T, Mistuna D, Dobrota D, Racay P. *Clin. Exp. Med.* 2009; 9:1–7. [PubMed: 18818983]
- (28). Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. *J. Biol. Chem.* 1951; 193:265–75. [PubMed: 14907713]
- (29). St. Croix CM, Wasserloos KJ, Dineley KE, Reynolds IJ, Levitan ES, Pitt BR. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2002; 282:L185–92. [PubMed: 11792622]
- (30). Zalewski PD, Fobes IJ, Seamark RF, Borlinghaus R.; Betts WH, Lincoln SF, Ward AD. *Chem. & Biol.* 1994; 1:153–161. [PubMed: 9383385]
- (31). Rana U, Kothinti R, Meeusen J, Tabatabai NM, Krezoski S, Petering DH. *J. Inorg. Biochem.* 2008; 102:489–99. [PubMed: 18171589]
- (32). Chiu Y, Canary JW. *Inorg. Chem.* 2003; 42:5107–5116. [PubMed: 12924881]
- (33). Sillen, LG.; Martell, AE. *Stability Constants of Metal-Ion Complexes, Special Publication No. 25.* The Chemical Society, Burlington House; London: 1971. p. 733-735.
- (34). Sillen, LG.; Martell, AE. *Stability Constants of Metal-Ion Complexes, Special Publication No. 25.* The Chemical Society, Burlington House; London: 1971. p. 623-632.
- (35). Lim NC, Freake HC, Bruckner C. *Chem. Eur. J.* 2005; 11:38–49. [PubMed: 15484196]
- (36). Tomat E, Lippard SJ. *Curr. Opin. Chem. Biol.* 2010; 14:225–230. [PubMed: 20097117]
- (37). Krezel A, Maret WJ. *Biol. Inorg. Chem.* 2008; 13:401–409.
- (38). Petering DH. *Chemtracts: Inorg. Chem.* 2004; 17:569–580.
- (39). Pearce LL, Wasserloos K, St. Croix CM, Gandle R, Levitan ES, Pitt BR. *J. Nutr.* 2000; 130:1467S–70S. [PubMed: 10801961]
- (40). Fahrni CJ, O'Halloran TV. *J. Am. Chem. Soc.* 1999; 121:11448–11458.

Figure 1. Cellular viability following 18 h exposure to TPEN with and without Zn²⁺.

Figure 2.

Time dependent loss of fluorescence following incubation with TPEN. (A) Zn^{2+} ($5\mu M$) from $ZnCl_2$ (50 mM Tris-Cl, pH 7.4), supernatant (50 mM Tris-Cl, pH 7.4), or cells (DPBS) was reacted first with $10\mu M$ TSQ to form fluorescent products and then with $100\mu M$ TPEN at $25^\circ C$. (B) Pseudo-first order rate constants for the reaction of $Zn(TSQ)_2$ ($5\mu M$) with TPEN were plotted vs [TPEN] to obtain rate constants for the reaction.

Figure 3.

Fluorescence emission spectra of LLC-PK₁ cells incubated with 30 μ M TSQ (blue) followed by addition of 30 μ M ZnCl₂ and 3 μ M PYR (green), and then 100 μ M TPEN (yellow, orange, red).

Figure 4.

Kinetics of quenching of Zinquin complexes by TPEN. (A) LLC-PK₁ cells (5×10^6 , approximately $5 \mu\text{M}$ total Zn^{2+}) were exposed to $10 \mu\text{M}$ ZQ_{EE} ($\diamond$) for 15 minutes before fluorescence was quenched with $100 \mu\text{M}$ TPEN. Result was compared with quenching of fluorescence of ZQ_{ACID}-Zn-proteome (black) and ZQ_{EE}-Zn-proteome (gray) with $100 \mu\text{M}$ TPEN. (B) Fluorescence decay after adding $100 \mu\text{M}$ TPEN to $5 \mu\text{M}$ Zn(ZQ_{ACID})₂ and $5 \mu\text{M}$ Zn(ZQ_{EE})₂. Inset: linear regression of k_{OBSERVED} vs. [TPEN] for the reaction of $5 \mu\text{M}$ Zn(ZQ_{EE})₂ with TPEN ($R^2 = 0.99$).

Figure 5.

Reactions of TPEN with reaction mixtures of ZQ_{ACID} and Zn_2 -ADH or Zn_2 -AP. (A) Decay kinetics of 5 μM ZQ_{ACID} - Zn_2 -ADH (gray) and ZQ_{ACID} - Zn_2 -AP (black) with 100 μM TPEN. Reaction mixtures of ZQ_{ACID} - Zn_2 -ADH (B) and ZQ_{ACID} - Zn_2 -AP (C) with TPEN were then chromatographed using Sephadex G-50 gel filtration. 5 μM Zn_2 -ADH (D) and Zn_2 -AP (E) were reacted with 100 μM TPEN for 30 minutes followed by separation by Sephadex G-50 gel filtration.

Table 1Intracellular distribution of Zn^{2+} (nmol/ 10^7 cells) following TPEN exposure.^a

Cellular Fraction	Zn^{2+} No Treatment ^b	Zn^{2+} 25 μM TPEN ^c	Zn^{2+} 100 μM TPEN ^b
Proteome	9.7 ± 0.49	8.8	6.8 ± 2.4
Metallothionein	3.8 ± 0.19	2.3	2.2 ± 0.21
Low molecular weight	2.0 ± 0.10	2.9	4.5 ± 2.8
Total	15 ± 0.78	14.0	14 ± 1.5

^a Conditions: Cells were treated for 30 minutes with the given concentration of TPEN prior to harvesting and fractionation of the supernatant.^b Average $\pm$ standard deviation (n = 3)^c Single experiment at 25 μM TPEN

Table 2Reactions of the LLC-PK₁ Zn-proteome with chelating agents and modified Zn-proteome with Zinquin^a

Competitive Ligand	Log K ^b	% Zn ²⁺ Chelation ^c	% Decrease in ZQ Fluorescence ^c
NTA	10.5 ³²	25±3	30±14
EGTA	8.8 ³³	25±3	51±23
TREN	14.4 ³²	26±2	43±7
EDTA	13.6 ³⁴	30±5	75±9
TPEN	15.4 ¹⁶	35±2	77±3

^aReaction conditions: Zn-proteome (10 μM Zn²⁺), treated for 30 min with 100 μM chelator in 20 mM Tris-Cl pH 7.4 buffer at 25° C.^b(conditional stability constant, pH 7)^cAverage ± standard deviation, n=3

Table 3Reaction of TPEN with mixtures of ZQ_{ACID} or ZQ_{EE} and Zn₂-ADH or Zn₂-AP

Reaction ^a	Zn _A	Zn _B
Zn ₂ -ADH + ZQ _{Acid}	Fluorescent Adduct (472 nm) ^b	Chelation as Zn(ZQ _{Acid}) ₂ (492 nm) ^b
Zn ₂ -ADH + ZQ _{Acid} + TPEN	Fluorescence Quench of Adduct (slower phase, Fig. 5A; no loss of Zn ²⁺) ^c	Fluorescence Quench of Zn(ZQ _{Acid}) ₂ (faster phase-Fig. 5A) ^c
Zn ₂ -ADH + ZQ _{EE} No chelation of Zn ²⁺ ; fluorescent adduct, 471 nm ²	---	---
Zn ₂ -ADH + ZQ _{EE} + TPEN	Fluorescence Quench of Adduct (Fig. 1s) ^c	Chelation as Zn-TPEN
Zn ₂ -ADH + TPEN	No chelation of Zn ²⁺ ^d	Chelation as Zn-TPEN
Zn ₂ -AP + ZQ _{Acid}	No Reaction	Chelated as Zn(ZQ _{Acid}) ₂ (492 nm) ^b
Zn ₂ -AP + ZQ _{Acid} + TPEN	No chelation of Zn ²⁺	Fluorescence Quench of Zn(ZQ _{Acid}) ₂ (fast, Fig. 5A) ^c
Zn ₂ -AP + ZQ _{EE} No chelation of Zn ²⁺ ; fluorescent adduct, 471 nm ²	---	---
Zn ₂ -AP + ZQ _{EE} + TPEN	Fluorescence Quench of Adduct (no loss of Zn ²⁺ , Fig. 1s) ^c	Chelation as Zn-TPEN
Zn ₂ -AP + TPEN	No chelation of Zn ²⁺ ^d	Chelation as Zn-TPEN

^aReaction conditions: [Zn₂-ADH] or [Zn₂-AP] = 5 μM; [ZQ_{Acid}] or [ZQ_{EE}] = 10 μM; [TPEN] = 100 μM in 20 mM Tris-Cl pH 7.4 buffer at 25° C.

^bλ_{MAX} of spectra; see Ref. 22

^cQualitative rate

^dEnzyme activity retained