Inorganic Chemistry

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Reaction of Metal-Binding Ligands with the Zinc Proteome: Zinc Sensors and *N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine

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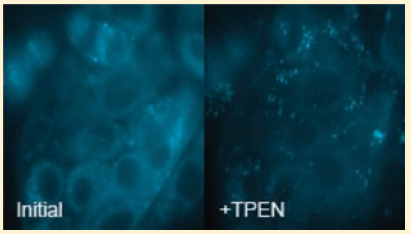
Supporting Information

ABSTRACT: The commonly used Zn^{2+} sensors 6-methoxy-8-*p*-toluenesulfonamidoquinoline (TSQ) and Zinquin have been shown to image zinc proteins as a result of the formation of sensor−zinc−protein ternary adducts not $Zn(TSQ)_2$ or $Zn(Zinquin)_2$ complexes. The powerful, cell-permeant chelating agent *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (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 the reaction of TPEN with cells pretreated with TSQ or Zinquin was not consistent with its reaction with $Zn(TSQ)_2$ or $Zn(Zinquin)_2$. Instead, TPEN and other chelating agents extract between 25 and 35% of the Zn^{2+} bound to the proteome, including $zinc^{2+}$ from zinc metallothionein, and thereby quench some, but not all, of the sensor−zinc−protein fluorescence. Another mechanism in which TPEN exchanges with TSQ or Zinquin to form TPEN−zinc−protein adducts found support in the reactions of TPEN with Zinquin−zinc−alcohol dehydrogenase. TPEN also removed one of the two Zn^{2+} ions per monomer from zinc−alcohol dehydrogenase and zinc−alkaline phosphatase, consistent with its ligand substitution reactivity with the zinc proteome.



INTRODUCTION

Zinc is an essential trace metal thought to be a cofactor 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 the 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]

N,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) 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]

$$Zn^{2+}{}_{TRANSIT} + TPEN \rightleftarrows Zn{-}TPEN \quad (1)$$

In the other, after incubation with a zinc 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]

$$Zn{-}sensor + TPEN \rightleftarrows Zn{-}TPEN + sensor \quad (2)$$

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 zinc proteins and, likewise, disturb the normal distribution of other metals such as iron and copper:

$$Zn{-}protein + TPEN \rightleftarrows Zn{-}TPEN + apoprotein \quad (3)$$

Second, it has been demonstrated recently that the commonly used Zn^{2+} sensors 6-methoxy-8-*p*-toluenesulfonamidoquinoline (TSQ) and Zinquin react primarily with Zn−proteins to form TSQ−Zn−protein ternary complexes not with intracellular Zn^{2+} to produce $Zn(TSQ)_2$.[21,22] TPEN quenches much of the fluorescence emission that results in the reaction of these Zn^{2+} sensors with cells. Previously, it was assumed that the reaction of TPEN with $Zn(TSQ)_2$ or $Zn(ZQ)_2$ 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 a 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

Received: November 22, 2011
Published: March 1, 2012

 © 2012 American Chemical Society dx.doi.org/10.1021/ic2025236 | *Inorg. Chem.* 2012, 51, 3625−3632

Zn^{2+} bound in the ternary complex or competes with TSQ or ZQ for binding to Zn−proteins:

$$\text{sensor}-\text{Zn}-\text{protein} + \text{TPEN} \rightleftarrows \text{Zn}-\text{TPEN} + \text{apoprotein} + \text{sensor} \quad (4)$$

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

$$\text{sensor}-\text{Zn}-\text{protein} + \text{TPEN} \rightleftarrows \text{TPEN}-\text{Zn}-\text{protein} + \text{sensor} \quad (5)$$

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-PK_1 cells were purchased from the American Tissue Culture Company. Medium 199 with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) growth media was supplemented with 50000 units/L Penicillin G, 50 mg/L streptomycin, and 4% fetal calf serum. Flasks were incubated under 5% CO_2 at 37 °C, and the medium was changed every 48 h. Cultures were subdivided by trypsin/ethylenediaminetetraacetate (EDTA) treatment every 5−7 days.

Reactions and Spectrofluorimetry of Cells or Cell Lysates Exposed to TSQ or ZQ Followed by TPEN. Exposures to *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), ZQ, 6-methoxy-8-*p*-toluenesulfonamidoquinoline (TSQ), pyrithione (PYR), or $ZnCl_2$ were conducted at concentrations that did not affect viability as measured by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium (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 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 47000*g* or 100000*g*. Cell suspensions or supernatants in the above buffer were added to a fluorescence cuvette. Their emission spectra were recorded between 400 and 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 Zinc 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, the sample was eluted by a gravity reservoir, and 20 drop fractions were collected. The void volume was ∼8 mL, and fractions 10−20 contained molecules larger than 10 kDa, referred to as the proteome. A subset of these proteins would be the zinc proteome. Fractions 20−30 included molecules of ∼10 kDa size, and fractions 30−40 were comprised of low-molecular-weight species. The 10 kDa Zn-containing species was identified as metallothionein (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 mixture of compressed air and acetylene. Measurements were obtained with a zinc 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+}, which correspond to 0.5, 1.0, and 2.0 μg/mL Zn^{2+}.

Reactions of TPEN with Model Zn−Proteins. Bovine erythrocyte alkaline phosphatase (AP) was obtained from Sigma. This protein, which binds two Zn^{2+} per monomer, was prepared to a concentration of 25 mg/mL by dissolution in degassed 50 mM HEPES and 0.1 M KNO_3 buffer, pH 7.2. Yeast alcohol dehydrogenase was purchased from Worthington Biochemical Corp. It contains two Zn^{2+} per monomer. The protein was prepared by dissolving the lyophilized powder in a 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 the 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 nicotinamide adenine dinucleotide (NAD) in 10 mM $Na_2P_2O_7$ buffer, pH 8.8, at 25 °C. The conversion of NAD to NADH was monitored at 340 nm for 30 s, and an initial rate was measured as the absorbance per minute. 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 s, and an initial rate was determined as the change in absorbance per minute.

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

RESULTS

Toxicity of TPEN and Zn−TPEN. LLC-PK_1 cells were completely viable after exposure to 100 μM TPEN for 1 h. However, 24 h of exposure reduced the cell viability to 30% (Figure 1). The effect was ameliorated by the addition of

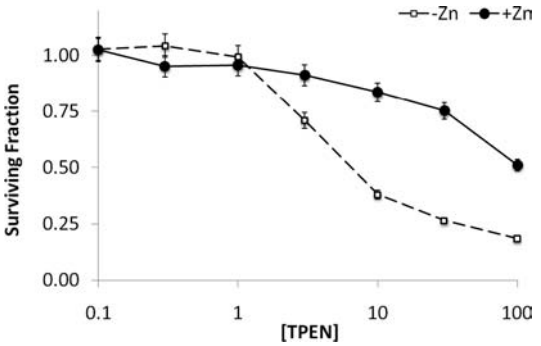


Figure 1. Cellular viability following 18 h of exposure to TPEN with and without Zn^{2+}.

equimolar $ZnCl_2$ 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-PK_1 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 μM Zn^{2+} with 100 μM TPEN resulted in a 90% loss of the fluorescence signal in the 7 min that followed first-order kinetics with a k_{obs} value of 2.7×10^{-3} s^{-1} (Figure 2A). λ_{max} of residual fluorescence in the TPEN-treated cells remained at 470 nm.

In a related experiment, cell supernatant containing 5 μM Zn^{2+} was mixed with 10 μM TSQ. The resulting fluorescence

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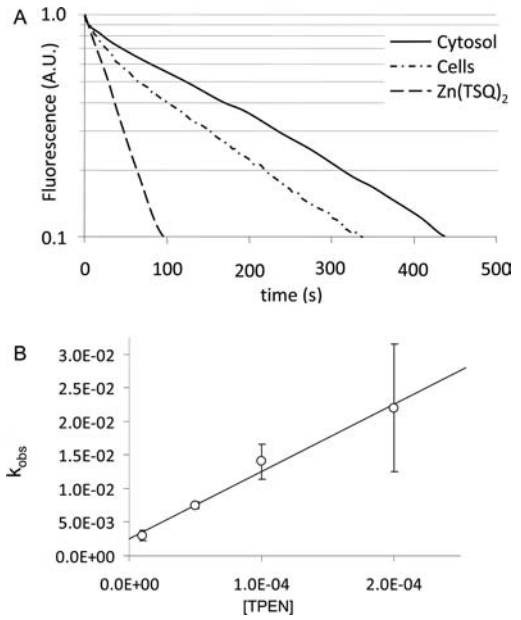

Figure 2. Time-dependent loss of fluoresence following incubation with TPEN. (A) Zn^{2+} (5 μM) from $ZnCl_2$ (50 mM Tris-Cl, pH 7.4), supernatant (50 mM Tris-Cl, pH 7.4), or cells (DPBS) was reacted first with 10 μM TSQ to form fluorescent products and then with 100 μM TPEN at 25 °C. (B) Pseudo-first-order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

emission spectrum exhibited λ_{max} at 470 nm, consistent with the formation of TSQ–Zn–proteins. The addition of 100 μM TPEN resulted in a first-order decay in fluorescence with a k_{obs} value of 2.1×10^{-3} s^{-1} (Figure 2A). Using 100 μM TPEN, cells and supernatant containing the same concentration of total Zn^{2+} and pretreated with TSQ reacted with similar pseudo-first-order kinetics.

For comparison, we examined the reaction of 100 μM TPEN with 5 μM $Zn(TSQ)_2$, the Zn^{2+} species that had previously been thought to account for TSQ-dependent cellular fluorescence. This reaction reached 90% completion in less than 100 s and was characterized by a pseudo-first-order rate constant of 2.5×10^{-2} s^{-1} (Figure 2A). According to these results, the induced loss of fluorescence caused by TPEN was 5-fold 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 μM) and the same concentration of Zn^{2+}. Because TSQ-incubated cells and an 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 the 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 μM $Zn(TSQ)_2$ and the formation of Zn–TPEN monitored by the loss of fluorescence. Plotting k_{obs} for the rate of $Zn(TSQ)_2$ decay against the concentration of TPEN revealed a linear relationship, with a slope of 99 s^{-1} M^{-1} representing the second-order rate constant (k_2) for part of the reaction and a nonzero intercept of 2.7×10^{-3} 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 Zn^{2+} ion prior to the rapid reaction of the remaining Zn–TSQ complex with TPEN.

Quenching of TSQ-Dependent Cellular Fluorescence by TPEN in the Presence of Extra Zn^{2+}. Another common tool in experiments about Zn^{2+} trafficking is the use of the ionophore PYR, which forms hydrophobic $Zn(PYR)_2$ complexes, to shuttle Zn^{2+} rapidly into cells. Incubation of 10^6 cells for 10 min with 3 μM PYR in the presence of 30 μM $ZnCl_2$ 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 to 490 nm, consistent with the formation of $Zn(TSQ)_2$ through the reaction of TSQ with $Zn(PYR)_2$ (Figure 3).[21] The addition of 100 μM TPEN reduced the

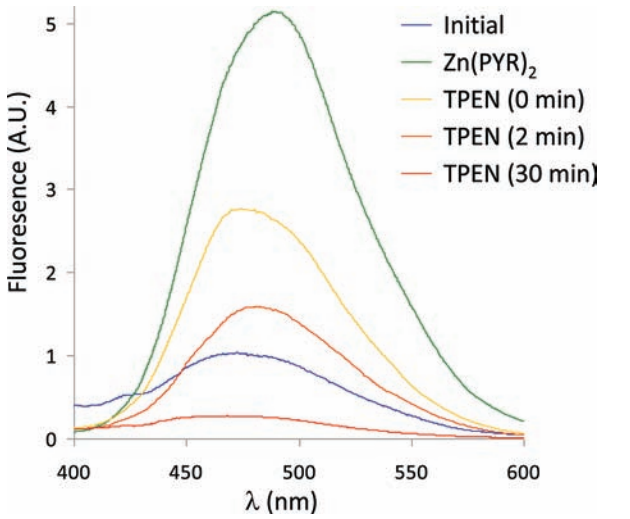

Figure 3. Fluorescence emission spectra of LLC-PK_1 cells incubated with 30 μM TSQ (blue) followed by the addition of 30 μM $ZnCl_2$ and 3 μM PYR (green) and then 100 μM TPEN (yellow, orange, and red).

fluorescence signal by nearly half within the time of mixing and then by about 50% again within 2 min. During this period, λ_{max} of the remaining signal moved toward 470 nm. Evidently, the rapid reaction represented the interaction of TPEN with $Zn(TSQ)_2$. The remaining fluorescence (77%) declined more slowly. The initial, swift reaction involved TPEN and intracellular $Zn(TSQ)_2$. 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 $Zn(TSQ)_2$ because neither its kinetics of reaction with TPEN nor its fluorescence emission spectrum matched the results of the reaction of TPEN with native cells or cell supernatant.

TPEN Quenching of Zn^{2+}-Dependent ZQ Fluorescence in LLC-PK_1 Cells and Zinc 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-PK_1 cells (approximately 5 μM total Zn^{2+}) pretreated with 10 μM Zinquin ethyl acetate [ethyl(2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy)acetate, ZQ_{EE}], the esterified sensor that rapidly accumulates in cells. Upon cellular uptake, ZQ_{EE} was assumed

dx.doi.org/10.1021/ic2025236 | Inorg. Chem. 2012, 51, 3625–3632

to hydrolyze to Zinquin acid (2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy)acetate, 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 multiphasic 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

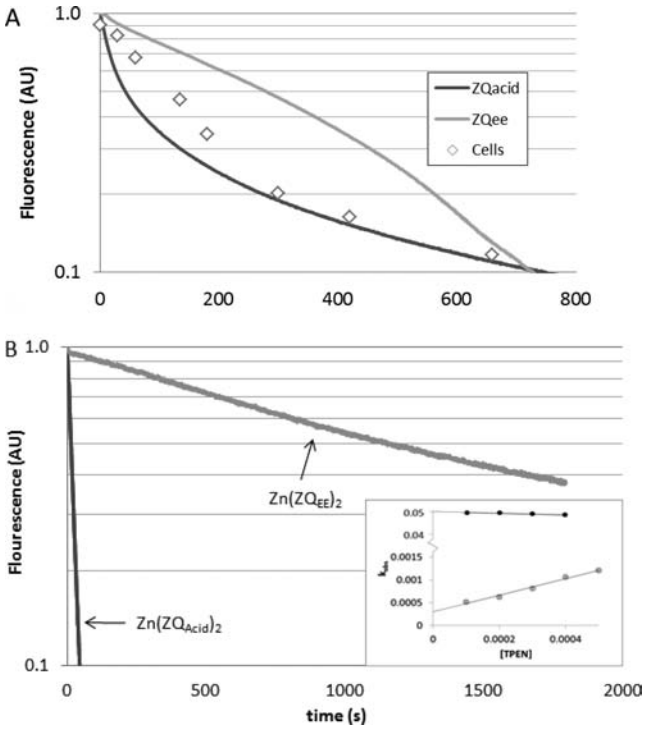

Figure 4. Kinetics of quenching of Zinquin complexes by TPEN. (A) LLC-PK_1 cells (5×10^6, approximately 5 μM total Zn^{2+}) were exposed to 10 μM ZQ_{EE} (◇) for 15 min before fluorescence was quenched with 100 μM TPEN. The result was compared with the quenching of fluorescence of ZQ_{ACID}–Zn–proteome (black) and ZQ_{EE}–Zn–proteome (gray) with 100 μM TPEN. (B) Fluorescence decay after the addition of 100 μM TPEN to 5 μM $Zn(ZQ_{ACID})_2$ and 5 μM $Zn(ZQ_{EE})_2$. Inset: linear regression of k_{obs} versus [TPEN] for the reaction of 5 μM $Zn(ZQ_{EE})_2$ with TPEN ($R^2 = 0.99$).

isolated zinc 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 kinetic profile qualitatively similar to that seen in the intact cells, although the major step involving about 70% of the fluorescence occurred somewhat faster than that in cells. ZQ_{EE} reacted more slowly. These results supported the hypothesis that TPEN rapidly accumulated in cells and then reacted with the ZQ_{ACID}–Zn–proteome as seen in vitro.

Reaction of TPEN with $Zn(ZQ_{EE})_2$ and $Zn(ZQ_{ACID})_2$. As models for the intracellular reactions of TPEN with ZQ-based complexes, the reactivity of TPEN with $Zn(ZQ_{EE})_2$ and $Zn(ZQ_{ACID})_2$ was determined. Like $Zn(TSQ)_2$, $Zn(ZQ_{ACID})_2$ 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 4.8×10^{-2} s^{-1}. Unexpectedly, the reaction of $Zn(ZQ_{EE})_2$ with TPEN proceeded at a much slower rate (Figure 4B). Moreover, it occurred with second-order kinetics, first-order in both $Zn(ZQ_{EE})_2$ and TPEN (k = 1.82 M^{-1} s^{-1}) with a nonzero y intercept of 3.0×10^{-4} s^{-1}.

The pseudo-first-order rates of reaction of TPEN with $Zn(TSQ)_2$ and $Zn(ZQ)_2$ 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 the kinetic behavior of $Zn(ZQ)_2$ species with TPEN was attributed to a much faster dissociation rate of ZQ_{ACID} from $Zn(ZQ_{ACID})_2$ than ZQ_{EE} from $Zn(ZQ_{EE})_2$ because of the electrostatic repulsion between the two negatively charged ZQ_{ACID} ligands.

Reaction of TPEN with Cellular Proteomic Zn^{2+}. Under standard culture conditions, LLC-PK_1 cells contained 16 ± 4.2 nmol of $Zn^{2+}/10^7$ cells. Incubation of these cells with 25 μM TPEN for 30 min prior to isolation of the cell supernatant did not significantly alter the total Zn^{2+} recovered (Table 1).

Table 1. Intracellular Distribution of Zn^{2+} (nmol/10^7 Cells) Following TPEN Exposure[a]

	Zn^{2+}		
cellular fraction	no treatment[b]	25 μM TPEN[c]	100 μM TPEN[b]
proteome	9.7 ± 0.49	8.8	6.8 ± 2.4
MT	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 min with the given concentration of TPEN prior to harvesting and fractionation of the supernatant. [b]Average ± standard deviation (n = 3). [c]Single experiment at 25 μM TPEN.

Quantifying the distribution of supernatant Zn^{2+} after Sephadex G-75 chromatography revealed that the proteomic Zn^{2+} band retained 90–100% of its total Zn^{2+}. The MT fraction was reduced by 60%, while the low-molecular-weight Zn^{2+} increased by 40%, indicative of the formation of Zn–TPEN. Increasing the concentration of TPEN to 100 μM, a concentration commonly employed in cellular experiments, reduced proteomic Zn^{2+} by 30%.[29,30] This reduction was accompanied by a 225% increase in low-molecular-weight Zn^{2+} thought to be Zn–TPEN. The small amount of Zn_7–MT (3.8 nmol/10^7 cells) typically present in LLC-PK_1 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 Zn^{2+} by TPEN. A published experiment showed that a series of ligands differing in the conditional stability constant for Zn^{2+} at pH 7 between 15.6 and 8.0 removes 20–30% of Zn^{2+} from the isolated Zn–proteome.[31] In the present experiment, Zn–proteome from LLC-PK_1 cells was reacted with a 10-fold excess of competing ligand (100 μM) for 30 min and the extent to ligand substitution transfer of Zn^{2+} from the proteome to the ligand determined by Sephadex G-75 chromatography. Using TPEN, EDTA, tris(2-aminoethyl)amine (TREN), ethylene

dx.doi.org/10.1021/ic2025236 | Inorg. Chem. 2012, 51, 3625–3632

glycol−bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and nitrilotriacetate (NTA) as ligands spanning a 10^8 range in the conditional stability constant, we observed that each sequestered about 30% of the proteomic Zn^{2+}, suggesting 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 was detected when the native proteome was probed with Zinquin.

Table 2. Reactions of the LLC-PK$_1$ 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

[a]Reaction conditions: Zn−proteome (10 μM Zn^{2+}), 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. [c]Average ± standard deviation, n = 3.

The extent of suppressed fluorescence was qualitatively and inversely related to the stability constant as seen in Table 2. For example, the initial reaction of TPEN with Zn−proteome resulted in 75% loss in fluorescence, whereas NTA depressed the fluorescence only 35%. These findings implied that these ligands react with different subsets of Zn−proteome, which, in turn, are differentially involved in the binding of 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 Zn^{2+} 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 Zn^{2+} 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 Zn_2ADH or Zn_2AP 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 Zn^{2+} in each protein, assumed to represent one of the two Zn^{2+} ions in each protein monomer, as $Zn(ZQ_{ACID})_2$.[22] The fluorophore also formed a fluorescent adduct with the other Zn^{2+} in Zn−ADH (ZQ_{ACID}−Zn−ADH) but did not react with the second site in Zn−AP.[22]

$$3ZQ_{ACID} + Zn_A, Zn_B\text{−ADH} \rightleftarrows ZQ_{ACID}\text{−}Zn_A\text{−ADH} + Zn_B(ZQ_{ACID})_2 \quad (6)$$

$$2ZQ_{ACID} + Zn_A, Zn_B\text{−AP} \rightleftarrows Zn_A\text{−AP} + Zn_B(ZQ_{ACID})_2 \quad (7)$$

The reaction of 100 μM TPEN with a mixture of 5 μM Zn_2ADH and 20 μM ZQ_{ACID} quenched the fluorescence of $Zn(ZQ_{ACID})_2$ as well as ZQ_{ACID}−Zn−ADH but did not extract the second Zn^{2+} ion from ADH according to Sephadex G-75 analysis of the product mixture (Figure 5A,B). Moreover, reactions of 5 μM Zn_2−proteins with 100 μM TPEN removed only one Zn^{2+} (Figure 5D,E). This implied that TPEN replaced ZQ_{ACID} in a ternary complex with Zn_A−ADH:

$$ZQ_{ACID}\text{−}Zn_A\text{−ADH} + TPEN \rightleftarrows TPEN\text{−}Zn_A\text{−ADH} + ZQ_{ACID} \quad (8)$$

As expected, the addition of TPEN to the reaction mixture of Zn_2−AP and ZQ_{ACID} also destroyed the resident fluorescence from $Zn(ZQ_{ACID})_2$ but, like ZQ_{ACID}, did not remove Zn_A^{2+} from Zn_A−AP (Figure 5A,C). Control reactions of TPEN with Zn_2−ADH and Zn_2−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 Zn_A−AP and Zn_A−ADH after chelation of Zn_B. In each case, the activities were diminished (27% for Zn_A−ADH and 19% for Zn_A−AP) compared to those of the holoenzymes. Because 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 ZQ_{ACID}−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] Because there is little, if any, free, aquated Zn^{2+}, it is assumed that the sensor, S, competes with zinc 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]

$$\text{zinc complex} + S \rightleftarrows Zn\text{−}S + \text{apo complex} \quad (9)$$

Depending on the changes in the 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 MT 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.):

$$TSQ + Zn\text{−protein} \rightleftarrows TSQ\text{−}Zn\text{−protein} \quad (10)$$

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 $Zn(PYR)_2$ to show that a source of Zn^{2+} would cause further enhancement in fluorescence. Finally,

dx.doi.org/10.1021/ic2025236 | Inorg. Chem. 2012, 51, 3625−3632

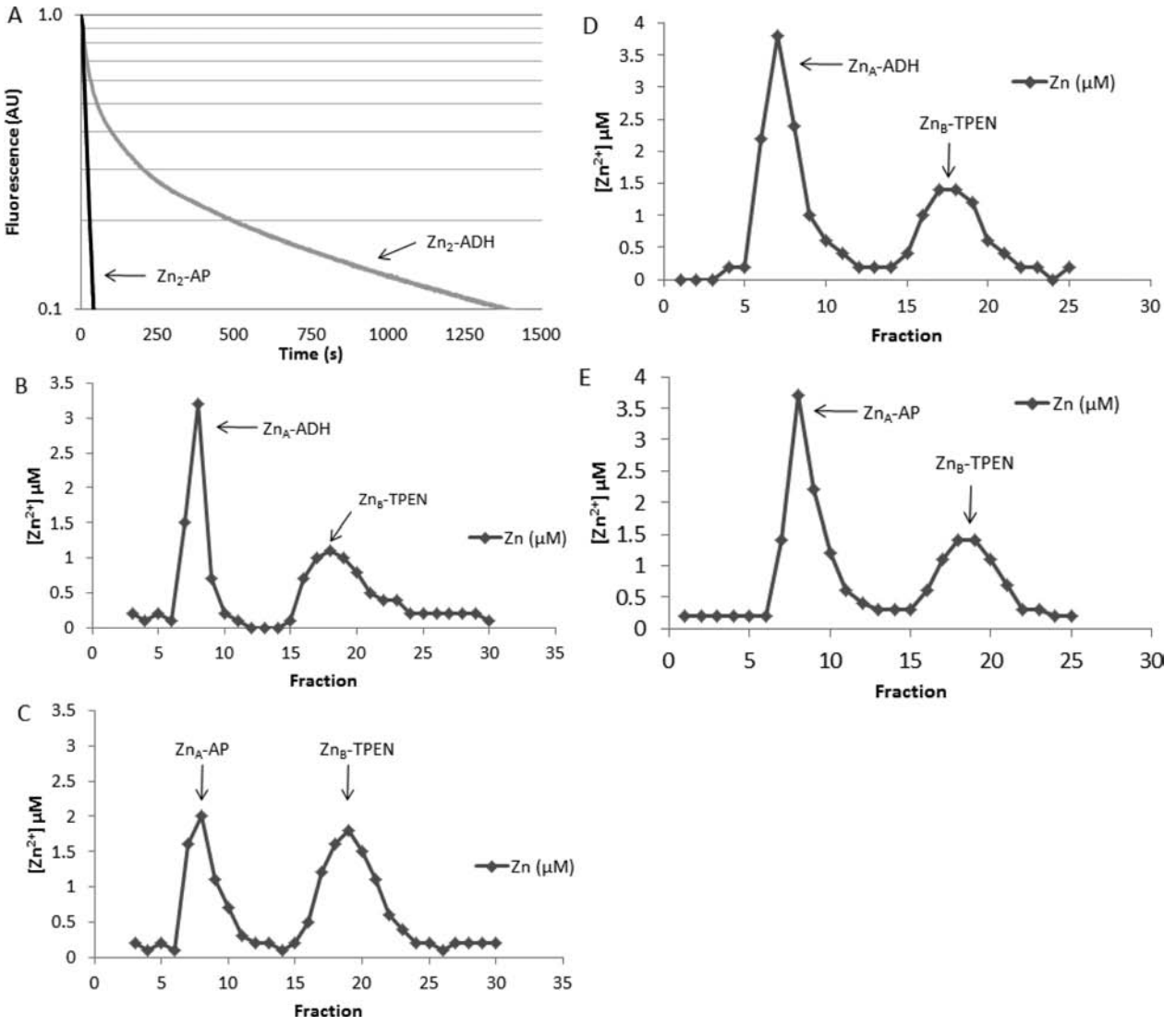

Figure 5. Reactions of TPEN with reaction mixtures of ZQ_{ACID} and Zn_2–ADH or Zn_2–AP. (A) Decay kinetics of 5 μM ZQ_{ACID}–Zn_2–ADH (gray) and ZQ_{ACID}–Zn_2–AP (black) with 100 μM TPEN. Reactions of mixtures of ZQ_{ACID}–Zn_2–ADH (B) and ZQ_{ACID}–Zn_2–AP (C) with TPEN were then chromatographed using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

TPEN, a powerful, cell-permeant chelating agent for Zn^{2+}, was supplied to quench the fluorescence and thereby confirm that fluorescence emission was due to the formation of the $Zn(TSQ)_2$ complex.[16]

$$Zn(TSQ)_2 + 2TPEN \rightleftarrows 2Zn{-}TPEN + 2TSQ \qquad (11)$$

It was assumed that all of the fluorescence enhancement following the addition of $Zn(PYR)_2$ resulted from the formation of $Zn(TSQ)_2$, 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] Reaction (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 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-PK_1 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 zinc 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 $Zn(TSQ)_2$ or $Zn(ZQ_{ACID})_2$ observed in vitro and in cells with $Zn(TSQ)_2$ (Figures 2 and 4). These results provided additional support for the conclusion that neither TSQ nor ZQ primarily imaged

dx.doi.org/10.1021/ic2025236 | Inorg. Chem. 2012, 51, 3625–3632

Table 3. Reaction of TPEN with Mixtures of ZQ_{ACID} or ZQ_{EE} and Zn_2−ADH or Zn_2−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; Figure 5A; no loss of Zn^{2+})[c]	fluorescence quench of $Zn(ZQ_{Acid})_2$ (faster phase; Figure 5A)[c]
Zn_2−ADH + ZQ_{EE}; no chelation of Zn^{2+}; fluorescent adduct, 471 nm[b]		
Zn_2−ADH + ZQ_{EE} + TPEN	fluorescence quench of adduct (Figure 1s in the Supporting Information)[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	chelation 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; Figure 5A)[c]
Zn_2−AP + ZQ_{EE}; no chelation of Zn^{2+}; fluorescent adduct, 471 nm[b]		
Zn_2−AP + ZQ_{EE} + TPEN	fluorescence quench of adduct (no loss of Zn^{2+}; Figure 1s in the Supporting Information)[c]	chelation as Zn−TPEN
Zn_2−AP + TPEN	no chelation of Zn^{2+} [d]	chelation as Zn−TPEN

[a]Reaction conditions: [Zn_2−ADH] or [Zn_2−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. [b]λ_{max} of spectra; see ref 22. [c]Qualitative rate. [d]Enzyme activity retained.

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 $Zn(TSQ)_2$ or $Zn(ZQ_{ACID})_2$ 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-PK_1 cells confirm that TPEN undergoes extensive ligand substitution with members of the Zn−proteome including Zn−MT [reaction (4)] over time (Table 1). In the process, the reactivity of the residual Zn−proteome with ZQ_{ACID} declined 75% in comparison with untreated 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 the 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. However, 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 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 Zn−proteome. Neither TSQ nor ZQ_{ACID} mobilized Zn^{2+} out of the LLC-PK_1 proteome or the Zn−proteomes from a variety of other cell types.[22] The overall log conditional stability constant of $Zn(ZQ_{ACID})_2$ 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 the 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. However, 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 iron and significant fractions of proteomic and MT-bound copper (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 a 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 iron and copper pools and the formation of 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). 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 bystander in cells that serve

dx.doi.org/10.1021/ic2025236 | Inorg. Chem. 2012, 51, 3625−3632

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.

■ ASSOCIATED CONTENT

Supporting Information

Reactions of TPEN with ZQ_{EE}−Zn_2−ADH and ZQ_{EE}−Zn_2−AP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Funding was made possible by 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.

■ REFERENCES

(1) Andreini, C.; Banci, L.; Bertini, I.; Rosato, A. *J. Proteome Res.* **2006**, *5*, 196−201.
(2) Andreini, C.; Banci, L.; Bertini, I.; Rosato, A. *J. Proteome Res.* **2006**, *5*, 3173−3178.
(3) Vallee, B. L.; Auld, D. S. *Faraday Discuss.* **1992**, *93*, 47−65.
(4) Budde, T.; Minta, A.; White, J. A.; Kay, A. R. *Neuroscience* **1997**, *79*, 347−358.
(5) Lu, Y. M.; Taverna, F. A.; Tu, R.; Ackerley, C. A.; Wang, Y. T.; Roder, J. *Synapse* **2000**, *38*, 187−197.
(6) Prasad, A. S. *J. Am. Coll. Nutr.* **2009**, *3*, 257−265.
(7) Cole, C. R.; Lifshitz, F. *Pediatr. Endocrinol. Rev.* **2008**, *4*, 889−896.
(8) Walsh, C. T.; Sandstead, H. H.; Prasad, A. S.; Newberne, P. M.; Fraker, P. J. *Environ. Health Perspect.* **1994**, *102* (Suppl2), 5−46.
(9) Fraker, P. J.; King, L. E. *Annu. Rev. Nutr.* **2004**, *24*, 277−298.
(10) Fraker, P. J. *J. Nutr.* **2005**, *135*, 359−362.
(11) Fukada, T.; Kambe, T. *Metallomics* **2011**, *3*, 662−674.
(12) Daniel, H.; tom Dieck, H. *Biol. Chem.* **2004**, *385*, 571−583.
(13) Ho, E.; Dukovcic, S.; Hobson, B.; Wong, C. P.; Miller, G.; Hardin, K.; Traber, M. G.; Tanguay, R. L. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2011**, Epub.
(14) Zalewski, P. D.; Forbes, I. J.; Betts, W. H. *Biochem. J.* **1993**, *296*, 403−408.
(15) Arslan, P.; Di Virgilio, F.; Beltrame, M.; Tsien, R. Y.; Pozzan, T. *J. Biol. Chem.* **1985**, *260*, 2719−2727.
(16) Anderegg, G. *Chim. Acta* **1977**, *60*, 123−140.
(17) Lu, H.; Pang, W.; Hu, Y. D.; Yang, H. P.; Huang, C. Y.; Jiang, Y. G. *Nutr. Neurosci.* **2011**, *14*, 80−87.
(18) Shin, J. H.; Jung, H. J.; An, Y. J.; Cho, Y. B.; Cha, S. S.; Roe, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 5045−5050.
(19) Bernhardt, M. L.; Kim, A. M.; O'Halloran, T. V.; Woodruff, T. K. *Biol. Reprod.* **2011**, *84*, 526−536.
(20) Tang, Z. L.; Wasserloos, K. J.; Liu, X.; Stitt, M. S.; Reynolds, I. J.; Pitt, B. R.; St. Croix, C. M. *Mol. Cell. Biochem.* **2002**, *234*−*235*, 211−217.
(21) Meeusen, J. W.; Tomasiewicz, H.; Nowakowski, A.; Petering, D. H. *Inorg. Chem.* **2011**, *50*, 7563−7573.
(22) Nowakowski, A.; Meeusen, J. W.; Petering, D. H. *Inorg. Chem.* **2011**, *50*, 10124−10133.
(23) Lee, J. Y.; Kim, J. S.; Byun, H. R.; Palmiter, R. D.; Koh, J. Y. *Brain Res.* **2011**, *1418*, 12−22.
(24) Kruczek, C.; Görg, B.; Keitel, V.; Pirev, E.; Kröncke, K. D.; Schliess, F.; Häussinger, D. *Glia.* **2009**, *57*, 79−92.
(25) Devergnas, S.; Chimienti, F.; Naud, N.; Pennequin, A.; Coquerel, Y.; Chantegrel, J.; Favier, A.; Seve, M. *Biochem. Pharmacol.* **2004**, *68*, 699−709.
(26) Sauer, G. R.; Smith, D. M.; Cahalane, M.; Wu, L. N.; Wuthier, R. E. *J. Cell. Biochem.* **2003**, *88*, 954−969.
(27) Hatok, J.; Babusikova, E.; Matakova, T.; Mistuna, D.; Dobrota, D.; Racay, P. *Clin. Exp. Med.* **2009**, *9*, 1−7.
(28) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265−275.
(29) St. Croix, C. M.; Wasserloos, K. J.; Dineley, K. E.; Reynolds, I. J.; Levitan, E. S.; Pitt, B. R. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2002**, *282*, L185−L192.
(30) Zalewski, P. D.; Fobes, I. J.; Seamark, R. F.; Borlinghaus, R.; Betts, W. H.; Lincoln, S. F.; Ward, A. D. *Chem. Biol.* **1994**, *1*, 153−161.
(31) Rana, U.; Kothinti, R.; Meeusen, J.; Tabatabai, N. M.; Krezoski, S.; Petering, D. H. *J. Inorg. Biochem.* **2008**, *102*, 489−499.
(32) Chiu, Y.; Canary, J. W. *Inorg. Chem.* **2003**, *42*, 5107−5116.
(33) Sillen, L. G.; Martell, A. E. *Stability Constants of Metal-Ion Complexes*; Special Publication No. 25; The Chemical Society, Burlington House: London, 1971; pp 733−735.
(34) Sillen, L. G.; Martell, A. E. *Stability Constants of Metal-Ion Complexes*; Special Publication No. 25; The Chemical Society, Burlington House: London, 1971; pp 623−632.
(35) Lim, N. C.; Freake, H. C.; Bruckner, C. *Chem.—Eur. J.* **2005**, *11*, 38−49.
(36) Tomat, E.; Lippard, S. J. *Curr. Opin. Chem. Biol.* **2010**, *14*, 225−230.
(37) Krezel, A.; Maret, W. *J. Biol. Inorg. Chem.* **2008**, *13*, 401−409.
(38) Petering, D. H. *Chemtracts: Inorg. Chem.* **2004**, *17*, 569−580.
(39) Pearce, L. L.; Wasserloos, K.; St. Croix, C. M.; Gandley, R.; Levitan, E. S.; Pitt, B. R. *J. Nutr.* **2000**, *130*, 1467S−1470S.
(40) Fahrni, C. J.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1999**, *121*, 11448−11458.