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Reactions of the Fluorescent Sensor, Zinquin, with the Zinc-Proteome: Adduct Formation and Ligand Substitution

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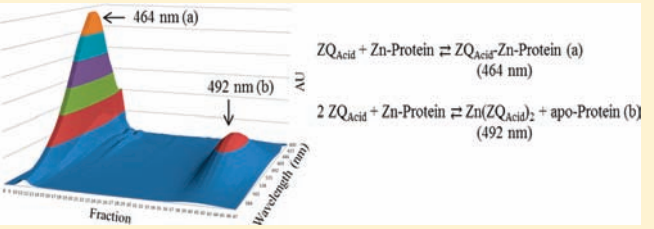
ABSTRACT: Zinquin (ZQ) is a commonly used sensor for cellular Zn^{2+} status. It has been assumed that it measures accessible Zn^{2+} concentrations in the nanomolar range. Instead, this report shows a consistent pattern across seven mammalian cell and tissue types that ZQ reacts with micromolar concentrations of Zn^{2+} bound as Zn-proteins. The predominant class of products were ZQ-Zn-protein adducts that were characterized in vivo and in vitro by a fluorescence emission spectrum centered at about 470 nm, by their migration over Sephadex G-75 as protein not low molecular weight species, by the exclusion of reaction with lipid vesicles, and by their large aggregate concentration. In addition, variable, minor formation of $Zn(ZQ)_2$ with a fluorescence band at about 490 nm was observed in vivo in each case. Because incubation of isolated Zn-proteome with ZQ also generated similar amounts of $Zn(ZQ)_2$, it was concluded that this species had formed through direct ligand substitution in which ZQ had successfully competed for protein-bound Zn^{2+}. Parallel studies with the model Zn-proteins, alcohol dehydrogenase (ADH), and alkaline phosphatase (AP) revealed a similar picture of reactivity: ZQ_{ACID} (Zinquin acid, (2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy)acetate)) able to bind to one Zn^{2+} and extract the other in Zn_2-ADH, whereas it removed one Zn^{2+} from Zn_2-AP and did not bind to the other. Zinquin ethyl ester (ethyl(2-methyl-8-*p*-toluenesulfonamido-6-quinolyloxy)acetate); ZQ_{EE}) bound to both proteins without sequestering Zn^{2+} from either one. In contrast to a closely related sensor, 6-methoxy-8-*p*-toluenesulfonamido-quinoline (TSQ), neither ZQ_{ACID} nor ZQ_{EE} associated with Zn-carbonic anhydrase. A survey of reactivity of these sensors with partially fractionated Zn-proteome confirmed that ZQ and TSQ bind to distinct, overlapping subsets of the Zn-proteome.



INTRODUCTION

The participation of Zn^{2+} in biological systems is both large and diverse. Bioinformatic analysis indicates that nearly 10% of the human genome codes for potential zinc-binding proteins.[1] The roles of Zn^{2+} in these proteins vary from structural stabilization as seen in Zn-finger transcription factors to involvement as a catalytic cofactor in hundreds of enzymes such as carbonic anhydrase and carboxypeptidase.[1,2] Besides these traditional roles, elevated intracellular Zn^{2+} concentrations can interact with other proteins to induce the synthesis of structures such as metallothionein.[3] Additionally, high levels of Zn^{2+} in synaptic vesicles and its mobilization under certain conditions have suggested that Zn^{2+} may act as a neurotransmitter.[4,5] Moreover, nutrient Zn^{2+} deficiency rapidly alters cell metabolism.[6,7] Thus, understanding Zn^{2+} distribution and trafficking within biological systems is central to grasping the contributions of Zn^{2+} to cellular metabolism.

Fluorescent probes have emerged as the researcher's main tool in studying basal and transient changes in labile Zn^{2+} in a variety of tissues.[8−15] Zn^{2+} sensors based on quinolinetoluenesulfonamide comprise a major class of such agents (Figure 1A). One of these probes is 6-methoxy-8-*p*-toluenesulfonamido-quinoline (TSQ), a fluorescent dye that binds to free Zn^{2+} in a 2:1 stoichiometry.[16] This molecule was first used as a histochemical stain to image Zn^{2+} within the central nervous system.[9] Another Zn^{2+} fluorophore, Zinquin or ZQ, has replaced TSQ in common use (Figure 1A and B).[13] It was introduced to overcome a perceived shortcoming of TSQ, namely, its zero charge that may permit cellular efflux of the probe. Moreover, because $Zn(TSQ)_2$ also bears no charge, it, too, might diffuse out of cells. In an attempt to circumvent these possible problems, an ethyl ester moiety was added to the methoxy group at the 8 position of the quinoline ring (Figure 1A). Like TSQ, ZQ_{EE}, the ethyl ester compound, readily permeates the cell membrane. However, once inside, it undergoes hydrolysis catalyzed by intracellular esterases to yield the negatively charged acid, ZQ_{ACID}, that ideally is fixed within the cell either as the free ligand or as $Zn(ZQ_{ACID})_2$ (Figure 1B). It should be noted, however, that this negatively charged species has been shown to have some degree of permeability through both plasma membranes and unilamellar liposomes.[17] In combination with the addition of the ethyl ester, the location of a methyl group at the 2 position enhances Zinquin's affinity for Zn^{2+} so that it may sense smaller intracellular fluxes of Zn^{2+} than TSQ.[16]

It has been assumed that sensors (S) such as TSQ and ZQ react with Zn-ligand complexes (Zn-L) to form fluorescent products:

$$\text{Zn-L} + 2\text{S} \rightleftarrows \text{Zn-S}_2 + \text{L(K, k)} \quad (1)$$

Zn-L includes Zn-proteins, small molecular weight complexes, and Zn^{2+}. The nature of Zn-L that is accessible to S depends on the

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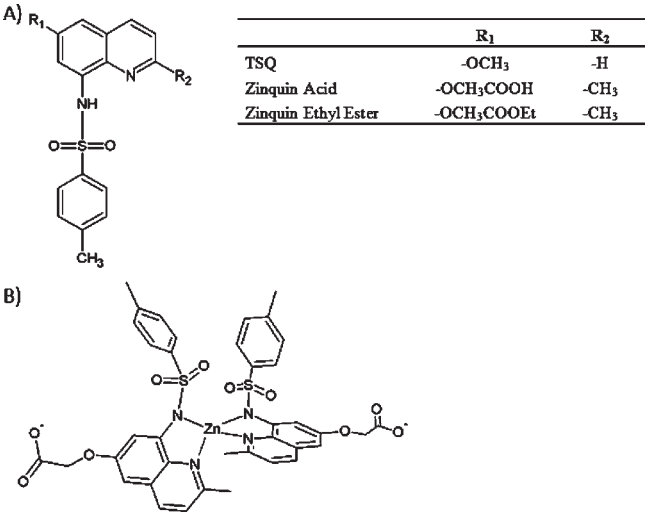

	R_1	R_2
TSQ	$-OCH_3$	-H
Zinquin Acid	$-OCH_3COOH$	$-CH_3$
Zinquin Ethyl Ester	$-OCH_3COOEt$	$-CH_3$

Figure 1. (A) Chemical structure of various quinoline-based fluorescent probes for sensing Zn^{2+}. (B) Chemical structure of $Zn(ZQ_{ACID})_2$.

thermodynamic (K) and kinetic (k) feasibility of reaction 1.[18] Despite their common use, little detail is known about the intracellular reactions of quinolinetoluenesulfonamide Zn^{2+} sensors.

Recently, experiments were undertaken to clarify the nature of the reactions of TSQ with cells.[19] It was determined that TSQ reacts primarily with components of the Zn-proteome by forming ternary adducts (reaction 2) not by generating $Zn(TSQ)_2$.

$$Zn\text{-}P + TSQ \rightleftarrows TSQ\text{-}Zn\text{-}P \quad (2)$$

Thus, TSQ images the cellular distribution of a subset of the Zn-proteome not Zn^{2+} or Zn^{2+} donated to TSQ through its reaction with Zn-proteins or other sources of Zn^{2+} (reaction 1).

It might be hypothesized that TSQ and ZQ_{ACID} behave similarly and form ternary adduct species with cellular Zn-proteins. However, some chemical properties of the two fluorophores differ from one another. ZQ_{ACID} binds Zn^{2+} several orders of magnitude more strongly than TSQ, a property that might favor the formation of $Zn(ZQ_{ACID})_2$.[20,21] Moreover, negatively charged ZQ_{ACID} might find different sites of reaction than TSQ. Thus, it was important to investigate the characteristics of reaction of ZQ_{EE} with cells and to compare them with TSQ. The present study demonstrates that while TSQ and ZQ_{EE} share themes of reactivity with cells, they differ in key ways. As a result, they are seen as complementary in their reactions with members of the Zn-proteome.

MATERIALS AND METHODS

Chemical Reagents. Zinquin ethyl ester (ZQ_{EE}) and zinquin acid (ZQ_{ACID}) were obtained from Enzo Life Sciences (Plymouth Meeting, PA). 6-Methoxy-(8-p-toluenesulfonamido) quinoline (TSQ) was obtained from AnaSpec (Fremont, CA). Sensors were solubilized in DMSO at stock concentrations of 12 mM and stored in the dark at 4 °C. All other chemicals were obtained from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific.

Cell Lines. All cell lines were purchased from the American Tissue Culture Company. Each was cultured as follows: pig kidney cells (LLC-PK1) in Medium 199 (Sigma) supplemented with 4% fetal calf serum (FCS); human medulloblastoma cells (TE671) in Dulbecco's Minimal Essential Growth-Low Glucose media (Sigma) supplemented with 5% FCS; human brain glioblastoma cells (U87 mg) in Eagles' Minimal Essential Media supplemented with 5% FCS; rat brain glioma cells (C6) in Ham's F-12 Nutrient Mixture-Kaighn's Modification media (Sigma) supplemented with 15% horse serum and 2.5% FCS; human epithelial lung carcinoma cells (A549) in Ham's F-12 Nutrient Mixture (Sigma) supplemented with 10% FCS; and human lymphocytic leukemia cells (CCRF-CEM) in suspension in RPMI 1640 media (Sigma) supplemented with 10% FCS. All media were additionally supplemented with 50 mg/L penicillin G and 50 mg/mL streptomycin. LLC-PK1, A549, and C6 cells were incubated in the presence of 5% CO_2 at 37 °C, while TE671, U87 mg, and CCRF-CEM cells were maintained in the presence of 6% CO_2 at 37 °C. Media were changed every 48−72 h until confluency was reached. Cultures were subdivided either by dilution in new complete media (CCRF-CEM) or by trypsin/EDTA treatment (LLC-PK1, U87 mg, TE671, C6, A549).

Mixed neuron-astrocyte cell suspensions from the cortex region—as well as the remainder of the brain—were isolated from fetal mice after 15−16 days of gestation. Pregnant mice were anesthetized with isoflurane and euthanized. Fetal brains from 7−8 pups were isolated in 3 mL of Eagles' Minimal Essential Media. Suspensions were prepared by mechanical tissue disruption and were washed 3 times in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) to further fragment the tissue. The final preparation contained about 90% neurons.

Fluorescent Spectroscopy of Cells. Cultured cells were grown in 100-mm culture plates to confluence. Culture media were decanted and plates were washed three times in cold DPBS. Cells were then suspended in DPBS by gently scraping with a rubber cell scraper. Fluorescent spectroscopy of the resultant suspension was performed using an Hitachi F-4500 fluorescent spectrophotometer. Emission spectra were recorded from 400 to 600 nm with an excitation wavelength of 370 nm using 10/5.0 nm excitation/emission slit widths. ZQ_{EE} (25 μM) was added to the suspension and allowed to incubate for 30 min and additional spectra were taken.[22]

Separation of Cell Lysates and Isolation of the Zn-Proteome. Cells were collected by centrifugation at 680*g* for 4 min, washed once in DPBS, and finally resuspended in 1 mL of cold double distilled H_2O. Cells were lysed via sonication and centrifuged at 47 000*g* for 20 min at 4 °C. The lysate—also referred to as the cell supernatant—was loaded onto an 80 cm × 0.75 cm Sephadex G-75 (GE Healthcare) gel filtration column and eluted with degassed 20 mM Tris-Cl, pH 7.4. Fifty 1-mL fractions were collected. Fluorescence spectra of all fractions were recorded as noted above. For cells not exposed to Zinquin, 10 μM ZQ_{ACID} was added to each fraction before fluorescence readings. The Zn^{2+} concentration of each fraction was determined by flame atomic absorption spectroscopy (AAS).

To isolate the Zn-proteome, unexposed cells were fractionated as described above. Fractions within the high molecular weight region containing absorbance at 280 nm were pooled and Zn^{2+} concentration was determined by AAS. The resultant pooled fractions were then referred to as the Zn-proteome.

Titration of Zn-Proteome with Zinquin. The initial fluorescence of the isolated Zn-proteome was recorded as the background fluorescence. Ten μM Zn-proteome was titrated with 1 μM ZQ_{EE}/ZQ_{ACID} with final additions of 2.5 and 5 μM to a final concentration of 20 μM ZQ. Reactions were incubated for 15 min between additions. After titration, the reaction mixture was loaded onto a 40 cm × 0.75 cm Sephadex G-50 (GE Healthcare) gel filtration column and eluted with degassed 20 mM Tris-Cl buffer, pH 7.4. Thirty 1-mL samples were collected. Fluorescence and Zn^{2+} concentrations of the eluted fractions were recorded as noted.

Approximately 10 μM (based on Zn^{2+}) isolated proteome from LLC-PK1 and TE671 cells was reacted with 0.1 mM EDTA for 30 min. The Zn-proteome was then run over a Sephadex G-50 column eluted with degassed 20 mM Tris-Cl buffer pH 7.4. Thirty 1-mL fractions were collected and reacted with 10 μM ZQ_{ACID} for 30 min before fluorescence and Zn^{2+} was determined as previously noted.

Table 1. Cell Models Used for Fluorescence Spectroscopy of Zinquin and Emission Properties of Exposed Cells

cell line, species	tissue	growth properties	Zinquin fluorescence[a]	λ_{max} (nm)
LLC-PK1, *Sus scofa* (pig)	kidney epithelial	adherent	39.8 ± 4.1	471
TE-671, *Homo sapien* (human)	medulloblastoma	adherent	165.1 ± 8.7	471
U87 mg, *Homo sapien* (human)	glioblastoma	adherent	153.2 ± 9.5	475
C6, *Rattus norvegicus* (rat)	brain glioma	adherent	78.9 ± 8.2	473
CCRF-CEM, *Homo sapien* (human)	T-cell lymphoblast	suspension	68.0 ± 1.9	471
A549, *Homo sapien* (human)	lung carcinoma	adherent	83.7 ± 10.8	473
mouse, *Mus muculus* (Swiss-Webster)	whole brain, > 9:1 neuron: astrocyte	suspension of primary dissection	46.7	474

[a] Five × 10^6 cells/mL, sum of fluorescence from 400 to 600 nm in thousand units, n = 3, except for mouse whole brain cells where n = 1.

Fluorophore Reactivity of Zn-Proteome from Te671 Cells. TE671 cells were collected, washed, and sonicated as previously noted. The sonicate was centrifuged at 100 000*g* for 40 min at 4 °C. The Zn-proteome was isolated by subjecting the resulting supernatant to centrifugation through a Millipore Centricon Centrifuge filter (30 000 MW cut off) at 4700*g* at 4 °C. The retentate was washed with degassed 20 mM Tris-Cl pH 8.0 and recentrifuged three times until the conductivity was that of the starting buffer. The Zn-proteome was loaded onto three conjoining Macro-Prep DEAE ion exchange columns (Bio-Rad) and eluted using degassed 20 mM Tris-Cl pH 8.0 with a salt gradient from 0 to 500 mM NaCl. One hundred 1-mL fractions were collected, divided in two, and initial background fluorescence was recorded. Fractions were then reacted with 5 μM ZQ_{ACID} or 5 μM TSQ for 30 min and additional spectra were taken and background fluorescence was subtracted.

The supernatant of sonicated TE671 cells after centrifugation at 100 000*g* for 40 min at 4 °C was loaded onto a 60 cm × 1.5 cm Sephacryl S-300 (Sigma) gel filtration column. The column was eluted with degassed 50 mM Tris-Cl pH 7.4 containing 150 mM NaCl. The flow rate was set for 1.0 mL/min and 110 1-mL fractions were collected. Each fraction was reacted with 5 μM ZQ_{ACID} for 30 min before fluorescence was recorded. To determine approximate molecular masses of the proteins within the elution fractions, gel filtration standards (Bio-Rad) containing thyroglobulin (670 000 Da), gamma-globulin (158 000 Da), albumin (44 000 Da), myoglobin (17 000 Da), and vitamin B12 (1350 Da) were run under the same elution conditions.

Titrations of Zinquin with Model Proteins. Yeast alcohol dehydrogenase (Worthington Biochemical Corporation) was prepared by dissolving the lyophilized powder in degassed 20 mM Tris-Cl buffer, pH 7.4. Bovine alkaline phosphatase (Sigma-Aldrich) was similarly prepared in degassed 50 mM HEPES buffer, pH 7.4. Bovine erythrocyte carbonic anhydrase (Sigma-Aldrich) was solubilized in degassed 50 mM Tris-Cl buffer, pH 8.0. After preparation, 10 μM carbonic anhydrase (Zn-CA), alkaline phosphatase (Zn_2-AP), or alcohol dehydrogenase (Zn_2-ADH) was titrated with 1 μM aliquots of ZQ_{ACID} or ZQ_{EE} until maximum fluorescence was reached. Samples were then eluted using Sephadex G-50 gel filtration. Fluorescence and Zn^{2+} concentration of eluted fractions were recorded as noted above.

RESULTS

Reactions of ZQ_{EE} with Cells. The fluorophore Zinquin was used in conjunction with fluorescence spectroscopy to detect Zn^{2+} within cultured cells. To investigate the generality of Zinquin-induced fluorescence, a variety of cell types were used in this study (Table 1). Because of the interest in Zn^{2+} distribution and trafficking in the brain, glioblastomal U87 mg and medulloblastoma TE671 cell lines were included as models for Zinquin exposure.[4,5,23] An immortalized pig kidney cell line (LLC-PK1) was examined because of previous reports of the reactivity of its Zn-proteome.[19,24] Microscopic studies of the interaction of Zinquin with C6 and A549 cells have been done previously, so these cell types were also used in this investigation.[10,12,15] A lymphocytic cell line, CCRF-CEM, which grows in suspension, was also examined for comparison with adherent cells.

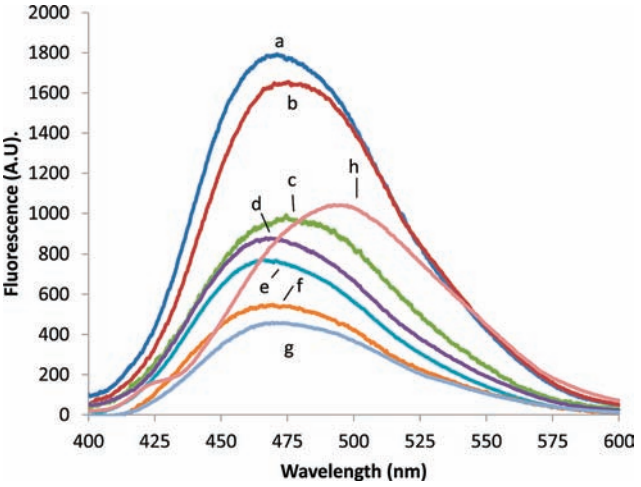

Figure 2. Fluorescence spectra of ZQ-exposed cells in comparison to 1 μM of $Zn(ZQ)_2$. Five × 10^6 TE671 (a), U87 mg (b), A549 (c), C6 (d), CCRF-CEM (e), mouse brain (f), and LLC-PK1 (g) cells were suspended in DPBS and reacted with 25 μM zinquin ethyl ester for 30 min before fluorescence was recorded using a 370 nm excitation. Spectra from all cell lines show emission profiles that are blue-shifted to 470 nm from the normal emission maximum of 492 nm for $Zn(ZQ)_2$ (h).

Figure 2 depicts the changes in fluorescence intensity of the different cell lines after exposure to ZQ_{EE}. The total fluorescence emission intensity normalized either to number of cells (5 × 10^6 cells/mL) or to protein content was unique to the cell type, suggesting Zinquin senses differing amounts of Zn^{2+} in each system. Nevertheless, all cell lines show emission spectra that place λ_{max} near 470 nm. These emission spectra are different from the expected emission spectrum of free Zn^{2+} bound to ZQ_{ACID}, which displays a λ_{max} of 492 nm (Figure 2h).[13] It is hypothesized that ZQ_{ACID} may react with open coordination sites of Zn-proteins to form ternary adducts instead of reacting with "free" Zn^{2+} to produce $Zn(ZQ_{ACID})_2$ resulting in emission spectra that are blue-shifted from 490 to 470 nm (reaction 3).[19,25]

$$ZQ_{ACID} + \textit{Zn-Protein} \rightleftarrows ZQ_{ACID}\textit{-Zn-Protein} \quad (3)$$

Fluorescence and Zn^{2+} Distribution of Exposed Cells. To investigate where the fluorescence is located within cells,

$Zinquin_{EE}$-exposed cell lysates were eluted using Sephadex G-75 gel filtration to separate the cellular components into three different pools:[19,26,27] the proteomic region containing molecules >10 kDa, metallothionein (MT) fractions (≈10 kDa), and the low-molecular-weight (LMW) region including molecules <10 kDa (Figure 3). Upon fractionation, nearly all of the fluorescence, thought to be due to the presence of ZQ_{ACID}, formed in situ from ZQ_{EE}, was confined to the proteomic region of the chromatogram. The emission spectra of these proteomic fractions were characterized by λ_{max} centered about 463 nm, displaying a major blue-shifted spectrum in comparison to $Zn(ZQ_{ACID})_2$. The spectrum was similar to what was seen with intact cells. In addition, almost all of the Zn^{2+} in the lysates eluted with proteomic fractions and not in the LMW pool. These results are consistent with the hypothesis that ZQ_{ACID} forms ternary ZQ_{ACID}-Zn-protein adducts that elute with the proteome fractions.

Fluorescence intensities of the proteomic fractions were converted to Zn^{2+} concentrations using a standard curve based on the fluorescence emission of $Zn(ZQ_{ACID})_2$. Comparing these values with the total Zn^{2+} concentrations measured by AAS shows that a differing amount of the proteome was reactive in each cell line (Table 2). The most reactive proteomes were those of the A549 cells and mouse brain, in which approximately 30% of the Zn^{2+} bound to the proteome appeared to coordinate with ZQ_{ACID}. Conversely, the least reactive proteome was that of the U87 mg cells in which only 15% of the Zn^{2+} was sensed by ZQ_{ACID}. Nevertheless, this range of values shows that substantial amounts of Zn^{2+} were accessed by ZQ_{ACID} in all cells that were tested.

Table 2 also reveals that there are some notable differences in Zn^{2+} concentration among the seven types of cells that were analyzed. C6 and TE671 diverge markedly from the others based on normalization to either cell number or protein content. Interestingly, the percentage of total Zn^{2+} in the proteome that was sensed by ZQ_{ACID} varied from 30 to 15%. This range was exhibited among the 5 cell types that displayed Zn^{2+} concentrations between 17 and 23 nmol/10^8 cells. Thus, either these cells contained markedly different sets of ZQ_{ACID}-reactive Zn-proteins or other factors controlled access of ZQ_{ACID} to these sites.

The U87 mg cell line was the only one that included a measurable amount of metallothionein (fractions 20−30 of Figure 3C). Metallothionein binds up to 7 Zn^{2+} ions, each with binding constants of about 10^{11}.[28] Unlike the proteome, the MT fractions did not show any fluorescence. Additionally, comparing ZQ_{EE} exposed and unexposed cells revealed that the amount of Zn^{2+} within these fractions was unperturbed under the two conditions (data not shown). When MT fractions from control cells were assayed with ZQ_{ACID}, no increase in fluorescence was observed over a 30 min period.

In some cases, LMW pools exhibited both fluorescence and Zn^{2+} ions. The extent of these pools varied between cell lines with TE671 cells containing the most fluorescence and Zn^{2+} and LLC-PK1 cells revealing no significant fluorescence or Zn^{2+} (Table 3). For the cell lines that contained LMW fluorescence, their spectra were identical with that expected for $Zn(ZQ_{ACID})_2$ with a λ_{max} centered around 492 nm (Figure 2h). In all cell lines, these pools were absent in unexposed cells that were first fractionated and then assayed with ZQ_{ACID} for fluorescence (data not shown). This control experiment demonstrated that in cells ZQ_{ACID} competed for proteomic Zn^{2+}, sequestering Zn^{2+}

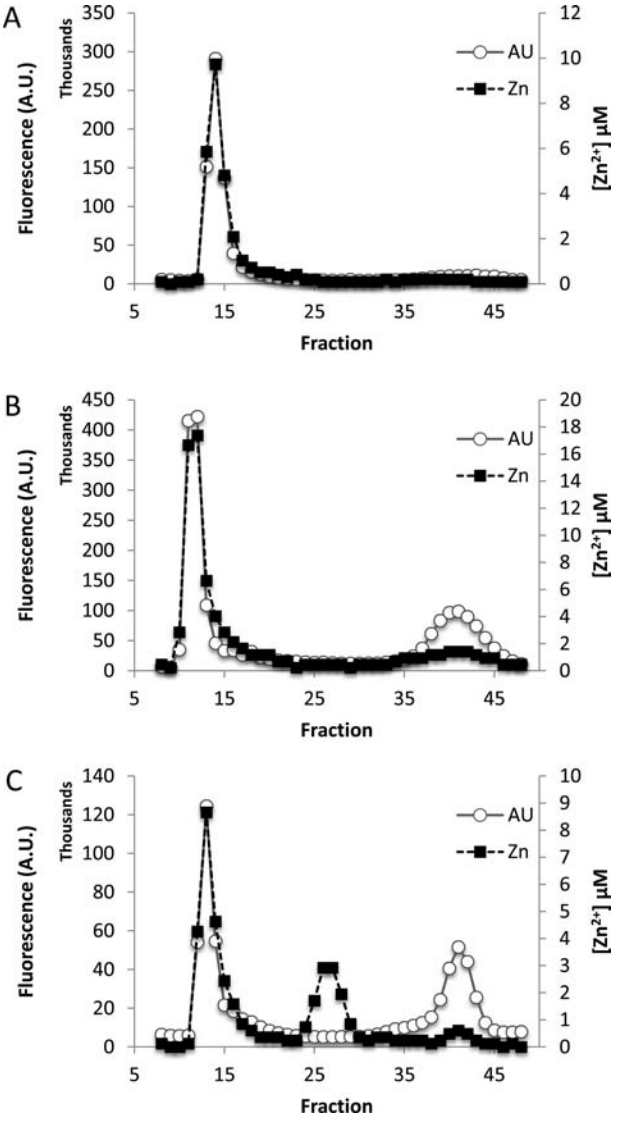

Figure 3. Fluorescence and Zn^{2+} distribution of Zinquin-exposed cells. 10^8 cells were incubated with 25 μM Zinquin ethyl ester before cytosolic fractionation via Sephadex G-75 gel filtration. Fractionation of LLC-PK1 (A) cells shows localization of fluorescence and Zn^{2+} solely to the proteomic region of the chromatogram with no pool of LMW Zn^{2+} or fluorescence present. The TE671 (B) cells show the largest pool of LMW Zn^{2+} or fluorescence in all cell lines tested. U87 mg (C) cells were the only cells tested that contained measurable amounts of metallothionein (fractions 20−30) which were unreactive to treatment with Zinquin. Note: Because emission spectra profiles are unique to Zn-species (see text), fluorescence measurements were calculated as the integrated intensity from 400 to 600 nm.

from the proteome and then eluting as $Zn(ZQ_{ACID})_2$ as proposed in reaction 4.

$$2ZQ_{ACID} + \textit{Zn-Protein} \rightleftarrows Zn(ZQ_{ACID})_2 + \textit{apo-Protein} \quad (4)$$

Parallel studies with TSQ, the parent compound of Zinquin, were conducted. Upon exposure of each cell type in Table 1 to TSQ followed by Sephadex G-75 fractionation of their cytosols, all of the fluorescence and Zn^{2+} was localized in the proteomic

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Table 2. Survey of Proteomic Zn^{2+} in Multiple Cell Types Exposed to 25 μM ZQ_{EE}

	Zn-proteome pool[a]				
cell line	Zn-AAS (nmoles)	fluorescence (AU)[b]	Zn-fluorescence (nmoles)[c]	% Zn reacted	λ_{max} (nm)
LLC-PK1[d]	23.6 ± 2.1	48.3 ± 12.6	4.2	18	462
C6[d]	12.5 ± 2.4	28.0 ± 3.0	2.5	20	463
TE671[d]	50.1 ± 4.9	112.2 ± 12.1	9.9	20	464
U87 mg[d]	18.6 ± 1.6	32.7 ± 4.7	2.8	15	462
CCRF-CEM[d]	17.4 ± 1.3	50.0 ± 9.1	4.4	25	463
A549[d]	17.1 ± 2.1	59.4 ± 15.5	5.1	30	465
mouse brain	18.8	67.1	5.9	31	463

[a] Per 10^8 cells. [b] Sum of fluorescence from 400 to 600 nm in ten thousand units. [c] Amount of Zn^{2+} sensed by fluorescence based on a standard curve of titration of ZQ_{ACID} with Zn^{2+}. [d] Average ± standard deviation of at least 3 replicates using different cell preparations.

Table 3. Survey of LMW Zn Pool in Multiple Cells Types upon Exposure to 25 μM ZQ_{EE}

	LMW Zn pool[a]			
cell line	Zn-AAS (nmoles)	fluorescence (AU)[b]	% of total Zn	λ_{max} (nm)
LLC-PK1[c]	0.6	5.9	2	
C6[d]	1.5 ± 0.3	10.0 ± 2.2	11	491
TE671[d]	11.1 ± 1.3	57.5 ± 6.2	18	492
U87 mg[d]	2.9 ± 0.6	19.8 ± 8.7	9	493
CCRF-CEM[d]	1.3 ± 0.4	17.8 ± 2.7	6	491
A549[d]	2.2 ± 0.6	19.0 ± 5.4	11	492
mouse brain	1.5	21.0	7	492

[a] Per 10^8 cells. [b] Sum of fluorescence from 400 to 600 nm in ten thousand units. [c] At limit of detection using flame atomic absorption spectroscopy. [d] Average ± standard deviation of at least 3 replicates using different cell preparations

fractions with emission peak maxima in the 470 nm range. Unlike ZQ_{ACID}, TSQ was not able to compete for Zn^{2+} in the proteomic region to form $Zn(TSQ)_2$.

Reactions of ZQ_{EE}/ZQ_{ACID} with the Zn-Proteome. Since proteomic Zn^{2+} was solely responsive to ZQ_{ACID} within cells, further characterization of the reaction of Zinquin with the isolated proteome was undertaken. The proteome of TE671 cells was isolated by size exclusion chromatography and reacted separately with ZQ_{EE} or ZQ_{ACID} (Figure 4). In both cases, the fluorescence after exposure displayed blue-shifted emission spectra with a λ_{max} of 470 nm for ZQ_{ACID} and 468 nm for ZQ_{EE}.

When the reaction mixtures were chromatographed, fluorescence was observed in both the proteomic region and the LMW regions. The LMW pools from the ZQ_{EE} and ZQ_{ACID} reactions were similar in both fluorescence intensity and Zn^{2+} concentration. These pools most likely represent reactive Zn^{2+} within the proteome that can undergo ligand substitution reaction with either Zinquin structure. The λ_{max} of both these pools is 492 nm, consistent with the formation of $Zn(ZQ_{ACID})_2$. Since the spectra maximum of $Zn(ZQ_{EE})_2$ is centered at 481 nm in a Tris buffer (data not shown), it appears that ZQ_{EE} had been hydrolyzed by proteomic esterases to form the acid and $Zn(ZQ_{ACID})_2$.

Chromatography of lysates from cells or isolated Zn-proteome treated with ZQ_{EE} demonstrated that most of the fluorescence was confined to the proteomic fractions. These fluorescent fractions again showed blue-shifted emission spectra with a λ_{max} of 468 and 466 nm for ZQ_{ACID} and ZQ_{EE}, respectively. However, the fluorescence for the ZQ_{EE}-reacted proteome was approximately 20% higher than the ZQ_{ACID}-exposed proteome. There are two possible reasons for this difference: (1) ZQ_{EE} reacted with more of the proteomic Zn^{2+} than ZQ_{ACID}. Because the binding moieties are the same, it is likely this difference is attributed to the negative charge on ZQ_{ACID} that may unfavorably interact with some Zn-proteins. (2) The quantum yield of ZQ_{EE}-Zn-protein adducts are greater than that for ZQ_{ACID}-Zn-protein adducts.

To test whether the reactive Zn^{2+} species in this pool labeled as the Zn-proteome was indeed Zn-proteins and not Zn^{2+} trapped within liposomal vesicles, the Zn-proteome isolated from LLC-PK1 cells was reacted with a 10-fold excess of EDTA (0.1 mM) and eluted over a G-50 size exclusion column. Because EDTA is not membrane permeant, any proteomic Zn^{2+} that undergoes ligand substitution with EDTA must be from kinetically labile, solvent-accessible sources and not from Zn^{2+} encased in vesicles. In agreement with a previously published result, EDTA chelated approximately 30% of the Zn^{2+} from the proteome that eluted as a Zn−EDTA complex in the LMW region.[24] When the reacted Zn-proteome was probed with Zinquin, the amount of fluorescence was 85% less than that of untreated Zn-proteome. Similar results were obtained using the proteome from TE671 cells. In that system, EDTA again chelated approximately one-third of the Zn^{2+} from the proteomic fraction. However, there was only a 45% depletion of fluorescence from the proteome versus control. In either case, residual fluorescence of the EDTA-treated proteomes does not necessarily mean it is from vesicular compartments, but rather from Zn-sources that are kinetically unfeasible for reaction with the potent chelator. In both EDTA-treated proteomes, the emission spectra were still centered at 470 nm.

These data support the claim that Zinquin interacts with a substantial concentration of soluble Zn-proteins. Moreover, since the Zn-proteome fraction was obtained from sonicated cells after centrifugation at 47 000*g* or 100 000*g* (next section) and either Sephadex G-75 or Sephacryl S-300 column chromatography, respectively, it is unlikely that liposomal vesicles, and certainly not native vesicles, remained in the Zn-proteome used in these experiments. The finding that the emission maximum of the fluorescent species was centered on 470 nm strongly supports the formation of ZQ-Zn-protein adducts because

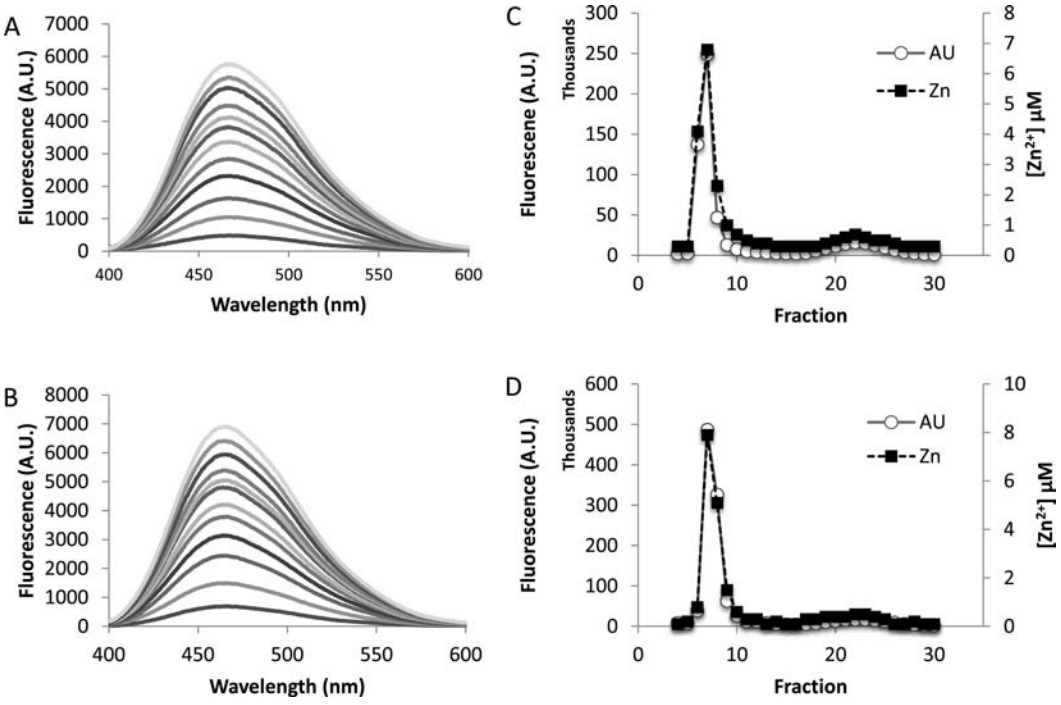

Figure 4. Titration of the Zn-proteome from TE671 cells with Zinquin acid and Zinquin ethyl ester. The 10 μM (based on Zn^{2+}) of the isolated proteome was titrated with increasing concentrations of Zinquin acid (A) and Zinquin ethyl ester (B) in 20 mM Tris-Cl pH 7.4 at 25 °C. The maxiumum emission of these titrations was centered at 470 nm, similar to what is seen in intact cells. Sephadex G-50 fractionation of the reaction product show that the fluorescense stays with the proteome with both Zinquin acid (C) and Zinquin ethyl ester (D).

interaction of $Zn(ZQ)_2$ with liposomes only causes a marginal blue-shifted spectrum.[17] In results to be presented elsewhere, we show that cytosol remaining after centrifugation at 100 000*g* was also reactive with ZQ. Thus, we conclude that ZQ reacted with Zn-proteins to form ternary, ZQ-Zn-protein adducts.

To further support that Zinquin reacted with cytosolic Zn-proteins, higher resolution gel filtration was used. The cell lysate of TE671 cells was centrifuged at 100 000*g* to remove not only cell fragments but any small organelles, such as microsomes, before separation by Sephacryl S-300 chromatography (Figure 5). The resulting chromatograph shows that fluorescence distributed throughout the eluted proteins. Moreover, a substantial amount of Zinquin fluorescence was associated with the molecular weight region from 100 000 to 10 000 Da, signifying reactivity with the Zn-proteins located within the supernatant. The emission spectra of this pool were again centered near 470 nm.

Reactions of ZQ_{EE}/ZQ_{ACID} with Model Zn-Proteins. To further investigate the reactivity of Zinquin with Zn-proteins, a model Zn-protein, alcohol dehydrogenase (Zn_2-ADH), was titrated with ZQ_{EE} and ZQ_{ACID}. ADH is a homodimer with each monomer containing 2 Zn^{2+} ions: one catalytic Zn^{2+} located within the active site and one structural Zn^{2+} that is solvent inaccessible.[2] As Zn_2-ADH was titrated with ZQ_{EE}, the fluorescence intensity increased with an emission spectrum maximum centered at 471 nm, consistent with the formation of a ZQ_{EE}-Zn_2-ADH ternary adduct (Figure 6). After eluting the reaction mixture using Sephadex G-50 gel filtration, indeed all fluorescence and Zn^{2+}were located in the proteome region. The fluorescence maximum remained at 471 nm, further supporting formation of a ternary adduct.

To determine any differences between the ethyl ester and acid forms, the experiment was repeated again, this time using

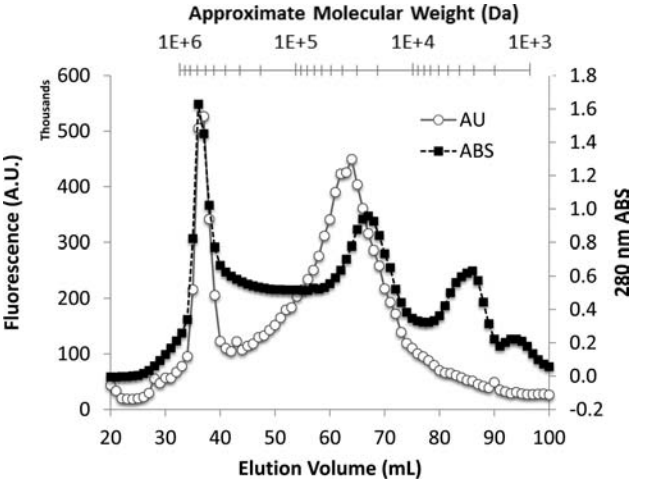

Figure 5. Sephacryl S-300 separation of TE671 cytosol. The cytosol of approximately 3 × 10^8 TE671 cells was run over a Sephacryl S-300 gel filtration column eluted with 50 mM Tris-Cl, 150 mM NaCl, pH 7.4. Fractions were assayed with 5 μM Zinquin acid. Approximate molecular weights of eluted fractions were determined using gel filtration standards.

ZQ_{ACID}. Upon titration, the fluorescence again increased but with a λ_{max} centered at 479 nm. Upon elution, two pools of fluorescence and Zn^{2+} were separated: one in the proteomic region and one in the LMW region. The proteomic region, containing half the total Zn^{2+}, exhibited a fluorescence spectrum that was blue-shifted to 472 nm, similar to that seen with ZQ_{EE}. The other half of the total Zn^{2+} was located in the LMW region and displayed a fluorescence spectrum characteristic of $Zn(ZQ_{ACID})_2$

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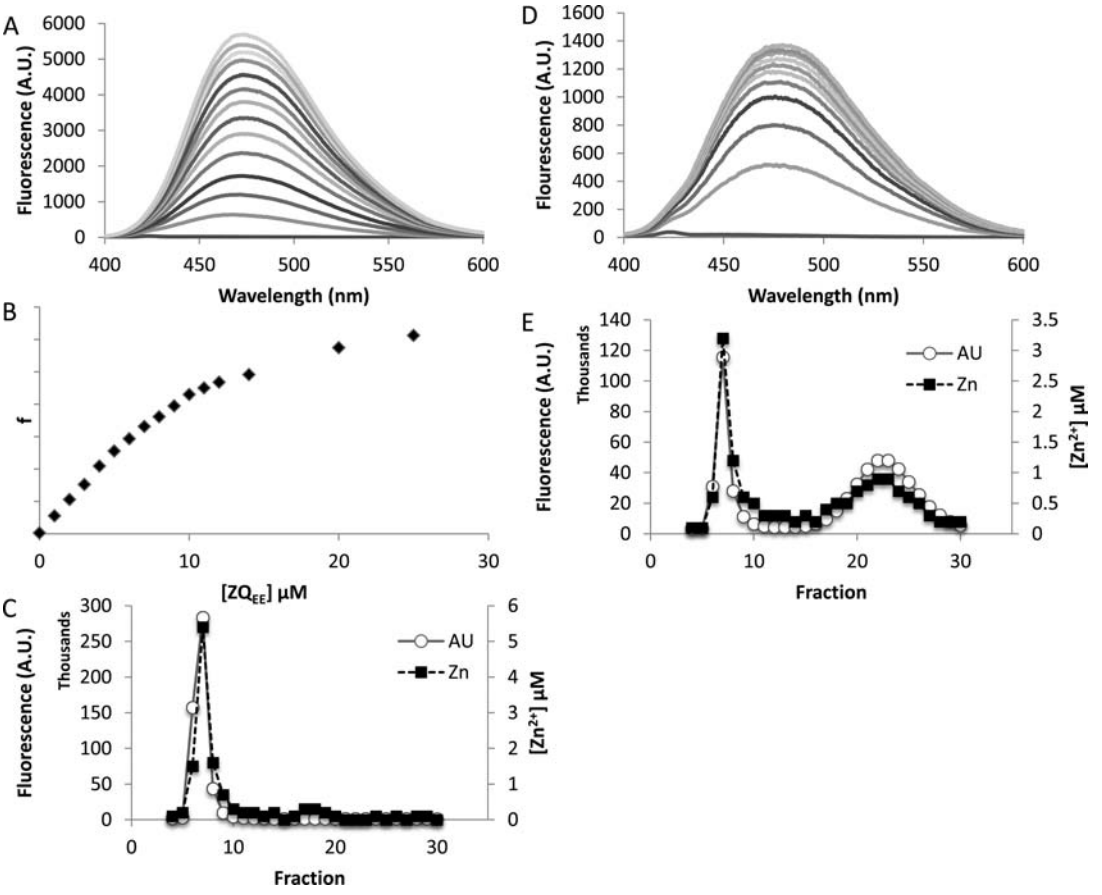

Figure 6. Zinquin reactivity with Zn_2-ADH. (A) Five μM Zn_2-ADH (10 μM Zn total) titrated with increasing amounts of Zinquin ethyl ester in 20 mM Tris-Cl pH 7.4 at 25 °C . (B) Fractional saturation vs concentration of Zinquin ethyl ester for the titration in Figure 5A. (C) Sephadex G-50 separation of the resulting titration in (A) showing fluorescence confined to the proteomic fractions. (D) Five μM Zn_2-ADH (10 μM total Zn^{2+}) titrated with increasing amounts of Zinquin acid in 20 mM Tris-Cl pH 7.4 at 25 °C. (E) Sephadex G-50 fractionation of 5 μM Zn_2-ADH with 10 μM Zinquin acid showing chelation of half of the Zn^{2+} from Zn_2-ADH.

centered at 492 nm. These data confirmed that ZQ_{ACID} not only can form a ternary adducts with Zn-proteins, but also was able to extract Zn^{2+} from native Zn-proteins. Further investigation is needed to determine which of the 2 Zn^{2+} centers in the protein underwent ligand substitution reaction with ZQ_{ACID}.

Titration with ZQ_{EE} was repeated with another Zn-protein, alkaline phosphatase. In this case, the fluorescence increased with a spectrum maximum at 475 nm, indicative of forming a ZQ_{EE}-Zn_2-AP ternary adduct. The observed dissociation constant for this adduct was 6.2×10^{-7} M (Figure 7). When the reacted protein was chromatographed, all the fluorescence and Zn^{2+} were confined to the proteomic fractions with the spectral maximum at 473 nm. These results provide additional support for the hypothesis that Zinquin can form ternary adducts with proteins.

When this titration was carried out with ZQ_{ACID}, the fluorescence spectrum was not blue-shifted and, instead, revealed the emission profile of free $Zn(ZQ_{ACID})_2$ complex. The chromatographed product showed that most of the fluorescence eluted in the LMW region; the residual fluorescence in the proteomic fractions was due to the natural fluorescence of Zn_2-AP. In addition, half of the Zn^{2+} eluted separately from the protein. These data indicate that ZQ_{ACID} chelated one of the two Zn^{2+} ions associated with Zn-AP, but was not reactive with the other Zn^{2+} still bound to the protein.

A third Zn-protein, carbonic anhydrase, was tested for reactivity with Zinquin. Previous work demonstrated that TSQ was able to bind Zn-CA strongly with a dissociation constant ca. 1.5×10^{-7} M.[19] When Zn-CA was titrated with either ZQ_{EE} or ZQ_{ACID}, no observable fluorescence increase was observed, indicating that Zinquin is not reactive with Zn-CA. A possible reason for this difference between the reactivities of Zinquin and TSQ with Zn-CA is that the methyl group at the 2 position of the quinoline ring on Zinquin sterically hinders the approach of Zinquin to the active site Zn^{2+}. Studies are ongoing to understand the structural basis of the different behavior of Zinquin and TSQ with Zn-CA.

Comparative Reactions of TSQ and ZQ_{ACID} with the Zn-Proteome. Because there appear to be differences between the reactivity of TSQ and Zinquin with both model proteins and the Zn-proteome, there may be differences within the sets of Zn-proteins that these fluorophores image within the cellular milieu. To test this hypothesis, the isolated Zn-proteome was further separated by anion exchange chromatography (Figure 8). Upon reaction of the resultant fractions with either ZQ_{ACID} or TSQ, the two fluorescence profiles were quite different. In some cases, fractions were fluorescent when assayed with either TSQ or ZQ_{ACID}. In other cases, fractions displayed reactivity with only one of the fluorophores. In either circumstance, the spectra of the reactive fractions were blue-shifted as seen in previous experiments. These data demonstrate that these fluorophores

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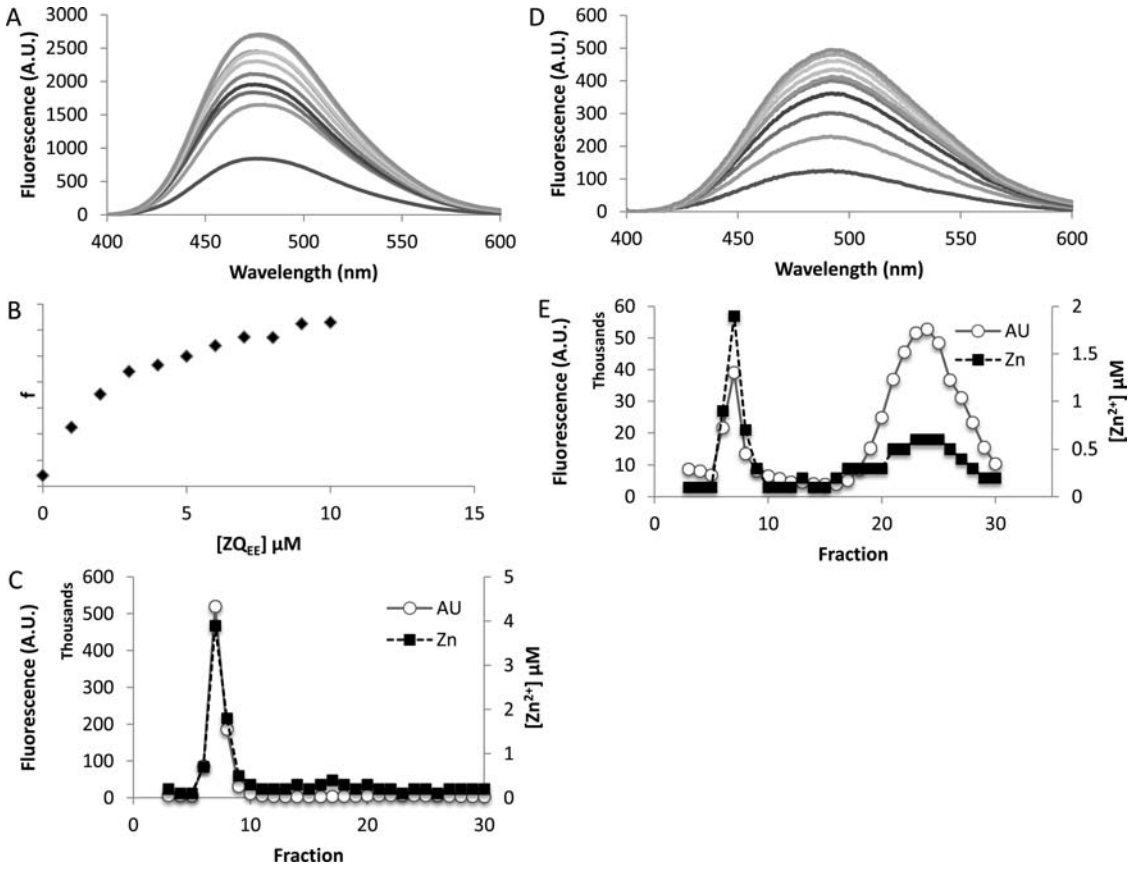

Figure 7. Zinquin reactivity with Zn_2-AP. (A) Five μM Zn_2-AP (10 μM Zn total) titrated with increasing amounts of Zinquin ethyl ester in 20 mM Tris-Cl pH 7.4 at 25 °C . (B) Fractional saturation vs concentration of Zinquin ethyl ester for the titration in Figure 6A. (C) Sephadex G-50 fractionation of the titration of Zn_2-AP with Zinquin ethyl ester showing fluorescence confined to the proteomic fractions. (D) Five μM Zn_2-AP (10 μM total Zn^{2+}) titrated with increasing amounts of Zinquin acid in 20 mM Tris-Cl pH 7.4 at 25 °C. (E) Sephadex G-50 fractionation of 5 μM Zn_2-AP with 10 μM Zinquin acid showing chelation of half of the Zn^{2+} from Zn_2-AP.

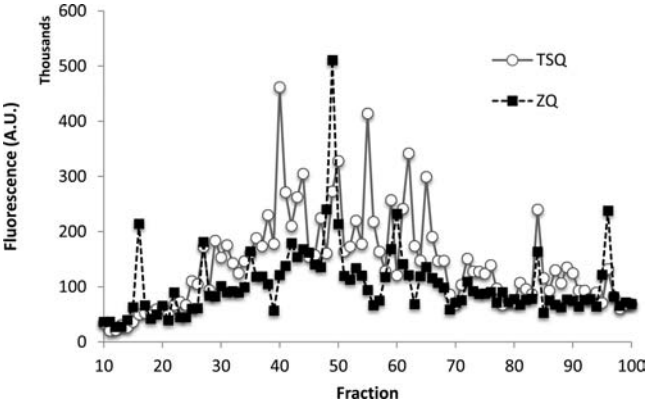

Figure 8. TSQ/ZQ reactivity with TE671 proteome. Isolated proteome was further separated by ion-exchange chromatography and reacted separately with 5 μM Zinquin acid (□) and TSQ (○). The resulting fluorescence pattern show that the each sensor reacts with differing subsets of the Zn-proteome.

are reactive with a different but overlapping subset of Zn-proteins within the Zn-proteome.

DISCUSSION

A variety of fluorescent sensors (S) have been synthesized with the aim to image cellular Zn^{2+}.[8,29] Mammalian cells contain μM concentrations of Zn^{2+}.[30] Almost all of it exists in the form of Zn-proteins, perhaps as many as 3000.[1] As a result, cellular *free* Zn^{2+} concentration resides in the high-pM to low-nM range.[31,32]

Conceptually, for reaction 3 to occur, both its equilibrium and kinetic properties

$$Zn\text{-}protein + S \rightleftarrows Zn\text{-}S + protein(K, k) \quad (5)$$

must be favorable. Thus, the operative language is that sensors react with *labile*, *loosely bound*, or *available* Zn^{2+}. These adjectives are sensor specific in that various sensors display a range of stability constants for Zn^{2+} and because of their different structures may be expected to undergo ligand substitution reactions with Zn-proteins at different rates.[18]

This perspective suggests that sensors may be able to react with members of the Zn-proteome. A recent study showed that between 20 and 35% of the proteomic Zn^{2+} from LLC-PK1 cells was reactive with competing ligands that form one-to-one complexes with Zn^{2+} when μM Zn^{2+} as Zn-proteome was reacted with near stoichiometric concentrations of ligands.[24] The apparent stability constants of these multidentate ligands in one-to-one complexes with Zn^{2+} ranged from 17.6 to 8.0. Thus, a significant fraction of proteomic Zn^{2+} may be available for ligand substitution reaction with sensors.

Beyond reaction 5, the multidentate nature of Zn^{2+} sensors suggests the possibility that adduct formation might result from

reaction of sensors with Zn-proteins. Many Zn-proteins are enzymes that utilize Zn^{2+} at the active site to coordinate one of the molecules undergoing catalytic reaction.[2] As such, they have open ligand binding sites that, in principle, could interact with the sensor to establish a ternary adduct species:

$$Zn\text{-}protein + S \rightleftarrows S\text{-}Zn\text{-}protein \quad (6)$$

A key paper by Hendrickson et al. demonstrated the propensity of the commonly used Zn^{2+} sensor, Zinquin, to form ternary complexes with small organic ligands.[25] The binding affinities of these Zinquin-Zn-adducts were comparable and some cases higher than that of a $Zn(ZQ)_2$ complex, which may lead to preferential adduct formation within a cellular environment. In addition, some adducts identified were characterized by fluorescence emission spectra that were blue-shifted in comparison with $Zn(ZQ_{ACID})_2$. Similarly, we have shown that the related sensor, TSQ, reacts in cells to form adduct species that are located in the proteome and characterized by blue-shifted spectra.[19] In this context, comparative experiments were undertaken with Zinquin to determine whether the behavior of TSQ extends to ZQ and how the differences in structure of the two sensors might alter their reaction with cells.

To elucidate the source of Zinquin-induced fluorescence, spectrofluorimetry was employed. This method provided greater detail than fluorescence microscopy about the nature of Zn^{2+} sites to which the fluorophore is bound. By measuring entire emission spectra for a large population of cells instead of the emission intensity of a few cells at a single wavelength, we observed that the emission spectra of a number of cell types exposed to ZQ_{EE} were different than the spectra of either $Zn(ZQ_{EE})_2$ or $Zn(ZQ_{ACID})_2$ (Table 1). All exhibited similar blue-shifted wavelength maxima near 470 nm in comparison to the reference spectra centered at 490 nm. The blue-shift was also observed with TSQ and is consistent with the formation of ternary adducts between ZQ and Zn-proteins.[19]

A companion result showed that the observed fluorescence represented 15−30% of all the cellular Zn^{2+} in the various cell types. Because previous estimates of total Zn^{2+} range among the hundreds of micromolar, it was evident that ZQ imaged micromolar not nanomolar concentrations of Zn^{2+}.[30,33] This conclusion implied that any hypothesis about the origin of the ZQ-based fluorescence must invoke the Zn-proteome pool as a significant source.

Cellular distribution of Zinquin fluorescence was briefly examined by Coyle et al. in primary liver extracts exposed to Zinquin in vitro.[26] In that system, fluorescence was found in not only the proteomic Zn^{2+} pool, but also in the MT and LMW pools. In contrast, Zn-MT did not react with Zinquin according to our experiments with intact cells as well as in vitro additions of Zinquin to the metallothionein pool. The discrepancy could possibly be accounted for by the previous use of an extremely high concentration of Zinquin (500 μM) in vitro as well as possible solvent effects due to a high concentration of DMSO (10%) employed in that work.

We tested the generality of our findings by surveying the reaction of ZQ_{EE} in a number of cell or tissue types. To our knowledge, this is the first extensive study investigating the intracellular targets of Zinquin in a variety of different cell types. Our exposure time and Zinquin concentration in buffered saline coincided with the parameters used in numerous microscopic experiments.[10,12−15,22,34] The measured fluorescence intensities of ZQ_{EE} treated cells indicated that micromolar concentrations of Zn^{2+} were being imaged in each cell type (Table 1). For the conversion of fluorescence intensities into concentrations, we used the standard curve for $Zn(ZQ_{ACID})_2$. Based on previous observations, adducts between ZQ_{ACID} and a number of Zn-ligand complexes exhibited emission quantum yields of the same order of magnitude as $Zn(ZQ_{ACID})_2$.[19,25] Other experiments have demonstrated that there are extremely low quantities of intracellular "free" Zn^{2+} that are in the nM−pM range.[30,32] The fact that we detected much more intense fluorescence emission supported our hypothesis that Zn-proteins not Zn^{2+} ions were the fluorescent species imaged by ZQ_{ACID}. This view was further corroborated by fractionating the cell lysates and testing their reactivity with EDTA to show that, indeed, the main source of Zinquin induced fluorescence resides in the proteome (Figures 3 and 5).

Zinquin reacted somewhat differently with each cell type. Upon reaction of ZQ_{EE} with kidney LLC-PK1 cells and the gel filtration fractionation of its supernatant, the only source of Zinquin fluorescence was within the proteome. However, in other cell types, not only was the proteome fluorescent, but generally small pools of free $Zn(ZQ_{ACID})_2$ were also isolated. Its concentration varied among the different cells that were assayed from 5 to 20% (Table 3). Because low-molecular-weight Zn^{2+} was not detected when unexposed cells were fractionated, this result indicated that Zinquin competed for protein bound Zn^{2+} as in reaction 7.

$$Zn\text{-}protein + 2ZQ_{ACID} \rightleftarrows Zn(ZQ_{ACID})_2 + apo\text{-}protein \quad (7)$$

This interpretation was supported when similar results were obtained in the reaction of ZQ_{EE} or ZQ_{ACID} with isolated Zn-proteome that did not contain any LMW Zn^{2+}.

According to our results summarized in reaction 7, Zinquin does not just "sense" free Zn^{2+} ions, it disturbs Zn^{2+} distribution within the cell. Interestingly, these low-molecular-weight pools of Zn^{2+} and fluorescence were not observed when cells were exposed to TSQ instead of ZQ_{EE}.[19] The 2:1 stability constant for TSQ for Zn^{2+} is approximately 10^{11} (unpublished result) and that of ZQ_{ACID} is about 10^{14}.[21] Apparently, the difference was sufficient to prevent TSQ from sequestering Zn^{2+} from the Zn-proteome. Thus, for TSQ, the Zn-proteome was not a source of labile or loosely bound proteomic Zn^{2+} as described in reaction 3; for ZQ_{ACID} it was.

The ability of Zinquin to bind to members of the Zn-proteome as well as to chelate Zn^{2+} from the proteome was further supported by in vitro reactions of Zn_2-ADH and Zn_2-AP with ZQ_{ACID}. With Zn_2-ADH, ZQ_{ACID} was able to form a ternary ZQ_{ACID}-Zn-protein adduct as identified by its blue-shifted fluorescence emission spectrum and comigration during gel filtration chromatography. Moreover, it competed for enzyme-bound Zn^{2+} to produce $Zn(ZQ_{ACID})_2$. ZQ_{ACID} also competed effectively for one of the two Zn^{2+} ions in Zn_2-AP. By analogy, the in vivo formation of $Zn(ZQ_{ACID})_2$ competes for protein-bound Zn^{2+} and may cause inactivation of functionally important Zn-proteins.

ZQ_{EE} displayed a different pattern of reactivity with Zn-proteome and model Zn-proteins than did ZQ_{ACID}. With isolated Zn-proteome as well as Zn_2-ADH and Zn_2AP, it formed fluorescent ternary adducts but did not successfully compete for Zn^{2+} to form $Zn(ZQ_{EE})_2$. The difference in structure between ZQ_{EE} and ZQ_{ACID} resides at the R_1 position of the isoquinoline ring in Figure 1. It is proposed that the negative charge of ZQ_{ACID}

favors the formation of $Zn(ZQ_{ACID})_2$ over ZQ_{ACID}-Zn-protein by destabilizing favorable binding interactions between ZQ_{ACID} and the protein environment surrounding the Zn^{2+} site.

The results presented here show that Zinquin, like TSQ, images Zn-proteins. Thus, the asymmetric distribution of intracellular fluorescence reported for Zinquin represents primarily the location of a subset of the Zn-proteome.[10,12,14,22,34−37] Nevertheless, the picture is more complicated than that for TSQ because some $Zn(ZQ_{ACID})_2$ also forms in cells exposed to ZQ_{EE}.

Further investigation of how TSQ and Zinquin react with the proteome shows that these fluorophores are not interchangeable with respect to Zn-protein association. After partial separation of the Zn-proteome by a combination of gel filtration and ion exchange chromatography, TSQ or ZQ_{ACID} were added to each fraction. The clear result was that the profiles of adduct formation activity were different. TSQ and ZQ_{ACID} do not necessarily react with the same Zn-proteins.

This conclusion was underscored in the experiments with model Zn-enzymes. Both TSQ and ZQ_{ACID} bind to Zn_2-ADH and Zn_2-AP. However, only TSQ reacts with Zn-CA; ZQ_{ACID} and ZQ_{EE} are unreactive with Zn-CA. A structural difference between ZQ and TSQ that could well impact their detailed sites of reaction is the presence of a 2-methyl group on the isoquinoline ring (Figure 1). Located next to the nitrogen atom that binds Zn^{2+}, the steric bulk of the methyl group might well serve as a barrier to adduct formation with some Zn-proteins. Because there appear to be substantial differences in binding specificity among these two sensors, conclusions drawn from microscopy studies using one fluorophore might not be the same if the other fluorophore had been used.

Zinquin binds to multiple Zn-proteins. It is plausible to suggest that adduct formation with open binding sites in Zn-proteins may interfere with the chemistries performed by those proteins. Initial studies of the reaction of ZQ_{ACID} with Zn_2-AP support this claim. Furthermore, Zinquin has an affinity for Cu^{2+} on the same order as that for Zn^{2+}, but the Cu^{2+} complex does not fluoresce.[13,21] Therefore, Zinquin may also be reactive with Cu-proteins in the same manner as Zn-proteins, but remain fluorescently invisible. Isolation and identification of ternary complexes with ZQ and TSQ need be performed to further understand how these sensors may be affecting metabolism during their residence in cells.

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