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Detection of Zn²⁺ release in nitric oxide treated cells and proteome: dependence on fluorescent sensor and proteomic sulfhydryl groups

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Nitric oxide (NO) is both an important regulatory molecule in biological systems and a toxic xenobiotic. Its oxidation products react with sulfhydryl groups and either nitrosylate or oxidize them. The aerobic reaction of NO supplied by diethylamine NONOate (DEA-NO) with pig kidney LLC-PK₁ cells and Zn-proteins within the isolated proteome was examined with three fluorescent zinc sensors, zinquin (ZQ), TSQ, and FluoZin-3 (FZ-3). Observations of Zn²⁺ mobilization from Zn-proteins depended on the specific sensor used. Upon cellular exposure to DEA-NO, ZQ sequestered about 13% of the proteomic Zn²⁺ as Zn(ZQ)₂ and additional Zn²⁺ as proteome•Zn–ZQ ternary complexes. TSQ, a sensor structurally related to ZQ with lower affinity for Zn²⁺, did not form Zn(TSQ)₂. Instead, Zn²⁺ mobilized by DEA-NO was exclusively bound as proteome•Zn–TSQ adducts. Analogous reactions of proteome with ZQ or TSQ *in vitro* displayed qualitatively similar products. Titration of native proteome with Zn²⁺ in the presence of ZQ resulted in the sole formation of proteome•Zn–ZQ species. This result suggested that sulfhydryl groups are involved in non-specific proteomic binding of mobile Zn²⁺ and that the appearance of Zn(ZQ)₂ after exposure of cells and proteome to DEA-NO resulted from a reduction in proteomic sulfhydryl ligands, favoring the formation of Zn(ZQ)₂ instead of proteome•Zn–ZQ. With the third sensor, FluoZin-3, neither Zn–FZ-3 nor proteome•Zn–FZ-3 was detected during the reaction of proteome with DEA-NO. Instead, it reacted independently with DEA-NO with a modest enhancement of fluorescence.

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Significance to metallicomics

A significant fraction of Zn²⁺ bound within the proteome other than zinc-metallothionein becomes reactive with zinc sensors upon exposure to NO, presumably as a result of sulfhydryl ligand modification. The native cell proteome also contains large numbers of adventitious binding sites for Zn²⁺ that hypothetically involve thiol ligands. In the presence of NO, the affinity of these sites is also reduced. As a result, the reactivity of various sensors with proteomic Zn²⁺ labilized by NO depends on their metal binding affinity in relation to the reduced affinity of the proteome, both native and adventitious binding sites, for Zn²⁺.

Introduction

Numerous zinc fluorescent sensors have been designed to provide researchers with tools to observe pools of intracellular Zn²⁺ that hypothetically participate in transient cellular events induced by normal or pathological conditions.^{1–3} In one of the well described reactions, elevated Zn²⁺ activates the MTF-1 transcription factor, leading to the induction of mRNA and the subsequent synthesis of metallothionein (MT) that sequesters excess intracellular Zn²⁺ and ZnT1 that transports Zn²⁺ from the cytosol into the extracellular medium.^{4–6} In addition, it has been proposed that Cd²⁺ stimulates MTF-1 binding to its

cognate DNA by displacing Zn²⁺ from basal MT, upregulating MTF-1, and eventually leading to the sequestration of Cd²⁺ by elevated concentrations of MT.⁶

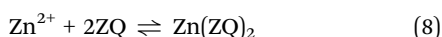
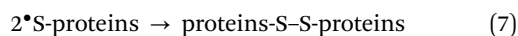
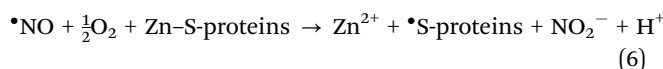
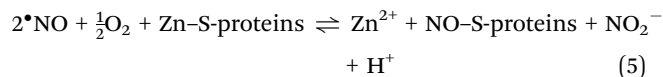
Nitric oxide (NO) serves both as a regulatory and toxic biomolecule, depending on the situation.^{7–10} Because NO and its oxidation products such as NO₂ and N₂O₃ display strong preference for reacting with sulfhydryl groups, past studies have investigated the reactivity of Zn–MT with these compounds, recognizing that MT contains 20 cysteinyl thiolate groups and uses them to bind up to 7 Zn²⁺ ions.^{11–13} For example, Pitt and coworkers hypothesized that NO exposure of lung endothelial cells releases Zn²⁺ from MT that subsequently causes vasoconstriction.¹⁴ Experiments supporting mobilization of Zn²⁺ were based on enhancement of fluorescence of the Zn²⁺ sensors zinquin and FluoZin-3 (FZ-3) in the presence of elevated NO production (Fig. 1).^{14–16}

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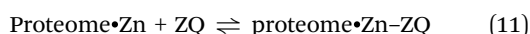
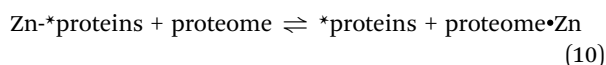
10 μM TPEN, a cell permeable, strong zinc chelator, quenched the fluorescence to 17% of the intensity observed with control cells treated with 20 μM ZQee (Fig. 2B), confirming that enhancement of fluorescence following incubation with ZQ_{ee} and DEA-NO involved the participation of Zn^{2+} . Moreover, the reaction of cells with DEA-NO reduced its total sulfhydryl concentration from 780 nmol/ 10^6 cells to 440 nmol/ 10^6 cells as measured after isolation of supernatant from sonicated cells.

The appearance of the shoulder at 490 nm suggested the mobilization of Zn^{2+} from the proteome (Zn-proteins) by NO or one of its oxidation products and, in turn, the production of the $\text{Zn}(\text{ZQ})_2$ complex (reactions (5)–(8)), which fluoresces with an emission maximum of 490 nm.^{19,21} It was hypothesized that oxidation products of NO modified Zn^{2+} -bound thiol ligands, thus releasing Zn^{2+} .²⁷



Another contributor to the fluorescence increase might be Zn^{2+} mobilized from Zn-proteins that non-specifically rearranges to adventitious binding sites within the proteome (proteome \cdot Zn) (reaction (3)). In turn, proteome \cdot Zn may react with ZQ to generate proteome \cdot Zn-ZQ that fluoresces at 470 nm (reaction (4)).¹⁹

To investigate further the basis for the above observations, 1.25×10^8 LLC-PK₁ cells in 10 mL were reacted with 20 μM ZQee for 30 min at room temperature before incubation with 500 μM DEA-NO for another 40 min. The cells were then lysed, centrifuged, and the resultant supernatant separated by Sephadex G-75 gel filtration. When the fractions were analyzed for fluorescence and zinc content, proteomic and low molecular weight (LMW) bands of both fluorescence and zinc were found for both control (cells plus ZQee) and DEA-NO exposed (cells and ZQee plus DEA-NO) cells (Fig. 3A and B). Proteome-associated fluorescence that centered at 470 nm increased by 16% in DEA-NO treated cells compared to control, unexposed cells. Evidently, DEA-NO induced the formation of more proteome \cdot Zn-ZQ adducts. Plausibly, the modification of SH groups by DEA-NO either made available altered native Zn-proteomic sites (Zn \cdot proteins) for reaction with ZQ or Zn^{2+} at such sites moved to non-specific proteomic sites where ternary adducts were generated (reactions (9)–(11)).



The low molecular weight fluorescence pool of control reaction constituted 19% of the total fluorescence (high molecular weight and low molecular weight), whereas that of DEA-NO treated reaction represented 32% (Fig. 3C). The spectra of low molecular

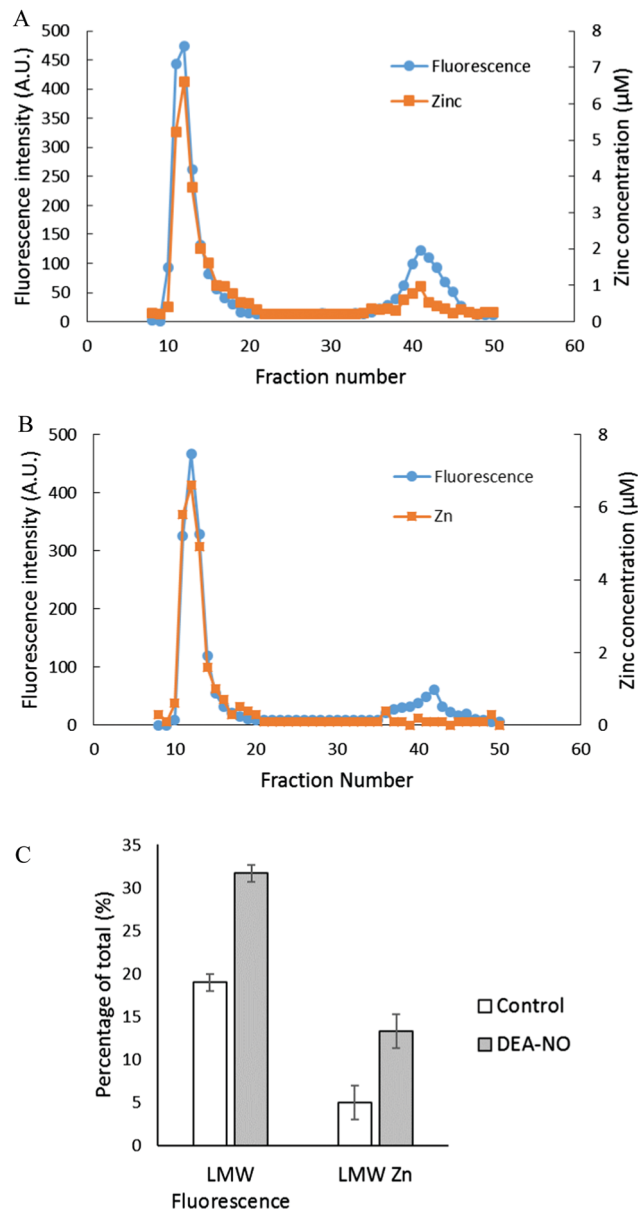
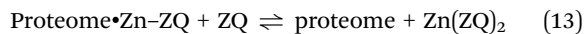
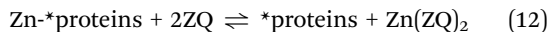


Fig. 3 Sephadex G-75 gel filtration of LLC-PK₁ cell supernatant incubated with 20 μM ZQee followed by 500 μM DEA-NO. (A) 10^8 LLC-PK₁ cells were reacted with 500 μM DEA-NO for 40 min following 20 μM ZQee for 30 min. The cells were lysed, centrifuged, and the supernatant separated using Sephadex G-75 column chromatography. The fractions were analyzed for both fluorescence and zinc. (B) Control: a parallel reaction was run under identical conditions without DEA-NO. (C) Comparison of the low molecular weight (LMW) fluorescence and zinc content between control and DEA-NO exposed cells. Error bars represent standard errors for at least three measurements.

weight fractions centered near 490 nm, the wavelength maximum of $\text{Zn}(\text{ZQ})_2$. Consistent with the fluorescence data, after DEA-NO treatment the low molecular weight zinc content was calculated to be 13% of the total zinc, whereas that of control reaction was only 5% (Fig. 3C). The significant increase of both the low molecular weight zinc and its accompanying fluorescence emission centered at 490 nm indicated the formation of $\text{Zn}(\text{ZQ})_2$ during the reaction of NO with cells. Possibly, ZQ extracted Zn^{2+} from native Zn^{2+}



binding sites that had been modified and weakened (Zn-*proteins) by nitric oxide to generate Zn(ZQ)₂ (reaction (12)). Alternatively, Zn²⁺ labilized at such sites may have shifted to non-specific sites of binding within the proteome (reaction (10)) and then reacted with ZQ to generate Zn(ZQ)₂ (reactions (11) and (13)):



Reaction of isolated proteome with DEA-NO in the presence of zinquin acid (ZQ_{acid})

The reactions of NO and ZQ with LLC-PK₁ cells were investigated in a simpler system that contained isolated proteome and was not complicated by membrane barriers and plentiful glutathione that are present in cells. Proteome containing 10 μM native protein-bound Zn²⁺ was first reacted with 20 μM ZQ_{acid} for 45 min at room temperature. As in whole cells, a gradual increase of fluorescence with the emission maximum of 470 nm was observed, indicative of the formation of ZQ-Zn-protein ternary adducts.²¹ Subsequently, 500 μM DEA-NO was added for an hour. When the final reaction mixture was separated by Sephadex G-75 chromatography, a noticeable low molecular weight pool of fluorescence and zinc, larger than that of the control sample, was detected (Fig. 4A and B). The LMW fluorescence pool (emission maximum of 490 nm) was measured to be 50% of total fluorescence, which is 1.7 times that of the control (30% of total fluorescence) (Fig. 4C) and displayed an emission maximum at 490 nm. In addition, the LMW zinc pool was determined to be three times larger than that of control proteome (15% of total zinc content *vs.* 5%). The reduction of sulfhydryl content by 41% (from 212 μM to 126 μM) following the treatment with DEA-NO demonstrated the reaction between proteomic sulfhydryl groups and nitric oxide. As in whole cells, the low molecular weight fluorescence and zinc pool in the reaction of isolated proteome indicated the production of Zn(ZQ)₂. Moreover, proteomic fluorescence intensity increased by 19% in comparison with the control, suggesting the generation of new ternary adduct sites that resulted from the labilization of Zn²⁺. Overall, the *in vitro* results agreed qualitatively with those derived from the exposure of cells to DEA-NO.

Reaction of LLC-PK₁ cells with DEA-NO in the presence of TSQ

The experiments involving ZQ were repeated substituting TSQ, its closely related analog (Fig. 1). First, TSQ was reacted with LLC-PK₁ cells to form TSQ-Zn-protein ternary adducts (reaction (1)) as was evident from the fluorescence spectrum centered at around 470 nm (Fig. 5A).¹⁸ In this bound form, TSQ like ZQ_{acid} is firmly fixed within the cells even though it is a neutral molecule. Subsequent incubation with 500 μM DEA-NO for 40 min caused a two-fold increase of fluorescence, substantially more than obtained with zinquin, and a shift of the emission maximum to 480 nm, which suggested that some Zn(TSQ)₂ had formed. As with ZQ, 10 μM TPEN was able to reduce the fluorescence to 32% of the control value, signaling the participation of Zn²⁺ in the enhancement of fluorescence in DEA-NO

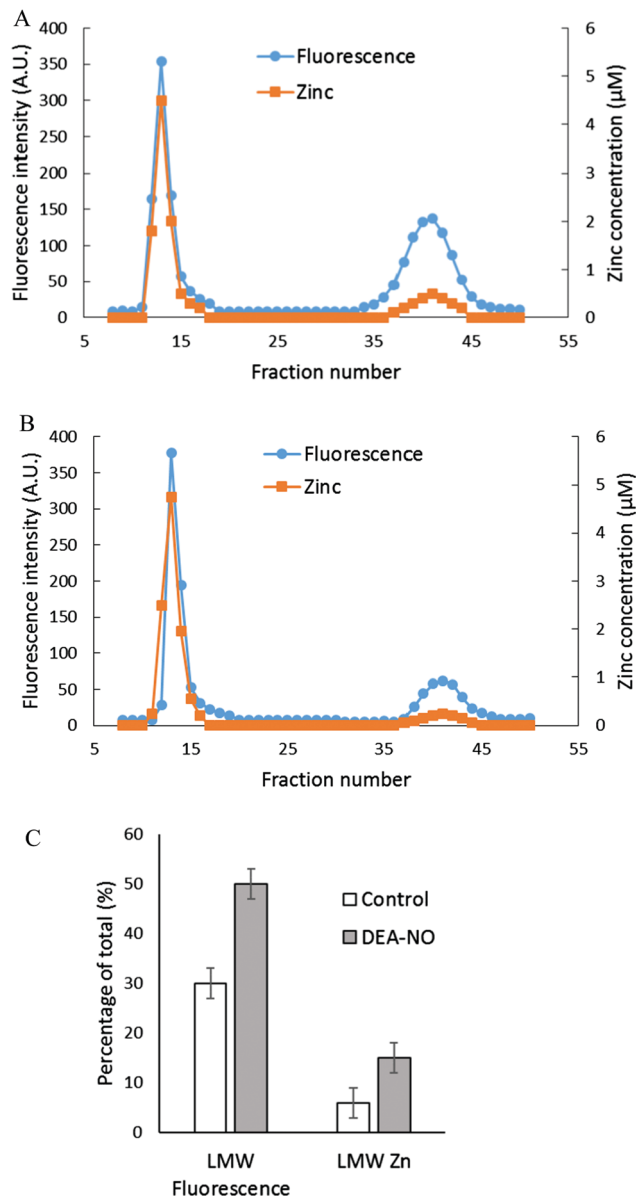


Fig. 4 Sephadex G-75 gel filtration of isolated proteome incubated with 20 μM zinquin acid (ZQ_{acid}) followed by 500 μM DEA-NO. (A) Isolated proteome (10 μM Zn²⁺) was reacted with 20 μM ZQ_{acid} for 45 min followed by 500 μM DEA-NO for another 1 hour. The final reaction mixture was separated using Sephadex G-75 column chromatography, and the fractions were analyzed for both fluorescence and zinc content. (B) Control: a parallel reaction was run under identical conditions in the absence of DEA-NO. (C) Comparison of the low molecular weight fluorescence and zinc content. Error bars represent standard errors for at least three measurements.

treated as well as control cells (Fig. 5B). Consistent with the fluorescence enhancement, the sulfhydryl concentration declined from 740 nmol/10⁶ cells to 450 nmol/10⁶ cells as measured after isolation of lysate from sonicated cells.

To characterize the fluorescent species, LLC-PK₁ cells were incubated with 20 μM TSQ for 40 min followed by 500 μM DEA-NO for another 45 min. Cells were then washed, lysed and centrifuged, and the resultant supernatant fractionated using



Sephadex G-75 column chromatography. Compared with the results with zinquin, no detectable pool of low molecular weight fluorescence and Zn^{2+} as $Zn(TSQ)_2$ was found in either DEA-NO treated or control cells (Fig. 6A and B). Almost all the fluorescence and zinc were detected in the high molecular weight proteome fractions. The fluorescence intensity of the high molecular weight fractions was 50% greater than that of control (Fig. 6C) and was aligned with the Zn^{2+} concentrations in the fractions. Moreover, the emission maxima of the proteomic fractions were located at about 470 nm, consistent with the presence of ternary adduct species. In contrast to the results in Fig. 5A, these findings indicate that Zn^{2+} mobilized by incubation with DEA-NO remained bound completely by the proteome in the presence of TSQ, forming ternary adducts instead of $Zn(TSQ)_2$. If some $Zn(TSQ)_2$ was formed *in vivo* as suggested by Fig. 5A, perhaps proteome•Zn-TSQ species became favored during chromatography as proteome and TSQ were separated and the reaction equilibrium, as in reaction (13), shifted to the left. That is, possibly because of its lower affinity for Zn^{2+} relative

to ZQ, TSQ cannot compete with the adduct species to retain $Zn(TSQ)_2$ during gel filtration.²⁰ However, a rapid one step separation of proteome from low molecular weight molecules using centrifugal filtration (3 kDa cut off Centricon filter) also did not demonstrate any $Zn(TSQ)_2$. Thus, it was concluded that mobilized Zn^{2+} was present only as proteome•Zn-TSQ.

Reaction of isolated proteome with DEA-NO in the presence of TSQ

The reaction of DEA-NO and TSQ with proteome was also examined (Fig. 6D). Isolated proteome containing 10 μM Zn^{2+} was reacted with 20 μM TSQ for 40 min before 500 μM DEA-NO was added for one hour. The reduction of sulfhydryl concentration by about 41% (from 340 μM to 200 μM) indicated a significant reaction between nitric oxide or its oxidation products and proteomic sulfhydryl groups. When the reaction mixture was subjected to Sephadex G-75 gel filtration, all of the fluorescence and Zn^{2+} resided in proteomic fractions; $Zn(TSQ)_2$ was not formed in either DEA-NO treated and control reactions.

Reaction of isolated proteome with added Zn^{2+} in the presence of ZQ or TSQ

The capacity of the proteome to bind adventitious Zn^{2+} in the absence or presence of DEA-NO and ZQ or TSQ was also examined in order to probe the involvement of reactions (2)–(4). Control proteome (8 μM Zn^{2+}) was titrated with Zn^{2+} in the presence of excess ZQ or TSQ. The fluorescence emission intensity centered at about 470 nm, due to the formation of proteome•Zn-ZQ/TSQ ternary adducts, increased in parallel with the concentration of added Zn^{2+} (Fig. 7A and 8A). In both cases, the emission maximum remained unchanged at 470 nm as Zn^{2+} was added to the proteome. Moreover, when the final reaction mixtures were fractionated by gel filtration with Sephadex G-75, no measurable low molecular weight fluorescence or Zn^{2+} was detected with either fluorophore (Fig. 7B and 8B). Thus, the proteome contained extensive capacity to bind Zn^{2+} and formed adducts with Zn^{2+} and either sensor (reactions (3) and (4)). These results differ from the findings about the reaction of cells or proteome with DEA-NO in the presence of zinquin. In those experiments, a red shifted emission maximum of the DEA-NO treated sample (Fig. 2) and a detectable low molecular weight fluorescence and zinc pool (Fig. 3 and 4) indicated that $Zn(ZQ)_2$ was produced along with proteome•Zn-ZQ.

The difference in outcome related to the absence or presence of DEA-NO. The results suggested the hypothesis that the behavior of ZQ depends on the existence and extent of modification of sulfhydryl groups that may participate in the adventitious proteomic binding of Zn^{2+} (reactions (14) and (15)).

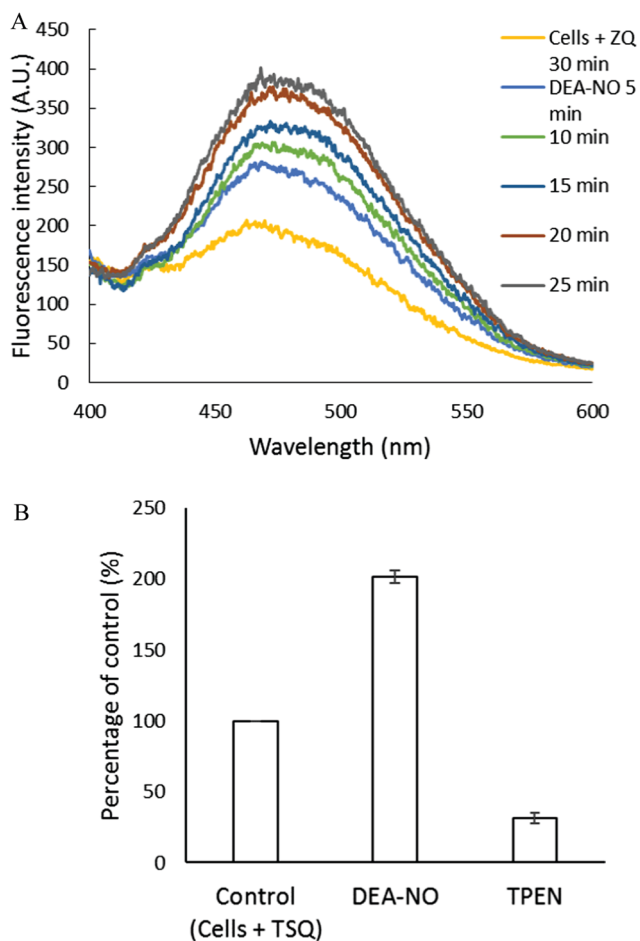
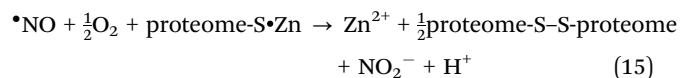
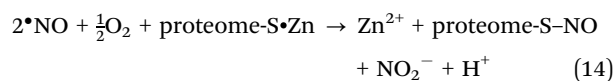


Fig. 5 Reaction of LLC-PK₁ cells with DEA-NO in the presence of TSQ. (A) Fluorescence spectra of the reaction between 10⁷ LLC-PK₁ cells and 20 μM TSQ for 30 min followed by the treatment with 500 μM DEA-NO for another 25 min. (B) Percent enhancement of fluorescence upon addition of 500 μM DEA-NO and its reversal by 10 μM TPEN. Error bars represent standard errors for at least three measurements.



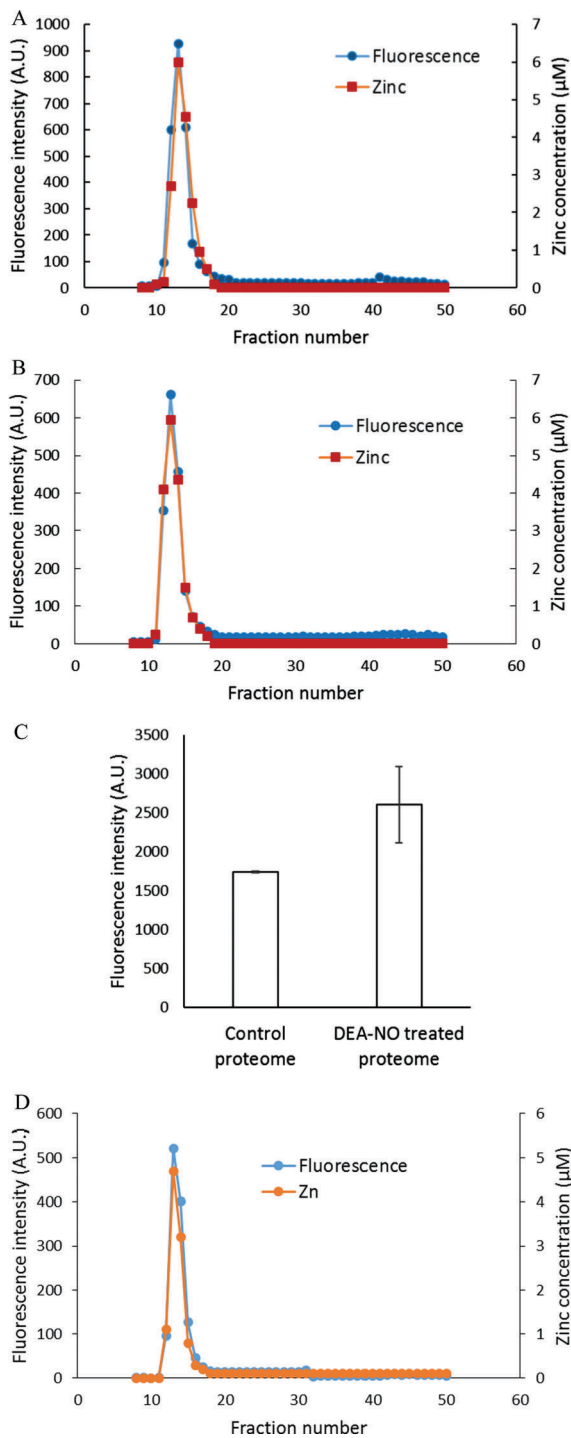


Fig. 6 Sephadex G-75 chromatography of LLC-PK₁ cell supernatant or isolated proteome incubated with TSQ followed by 500 μM DEA-NO. (A) 10^8 LLC-PK₁ cells were reacted with 500 μM DEA-NO for 45 min following 20 μM TSQ for 40 min. The cells were lysed, centrifuged, and the supernatant was separated using a Sephadex G-75 column. The fractions were analyzed for both fluorescence and zinc. (B) Control: a parallel reaction was run at identical condition with no DEA-NO added. (C) Comparison of the integrated fluorescence intensity of proteome. (D) Isolated proteome (10 μM Zn²⁺) was reacted with 20 μM TSQ for 40 min followed by 500 μM DEA-NO for another 1 hour. The final reaction mixture was separated using a Sephadex G-75 column, and the fractions were analyzed for both fluorescence and zinc content. Error bars represent standard errors for at least three measurements.

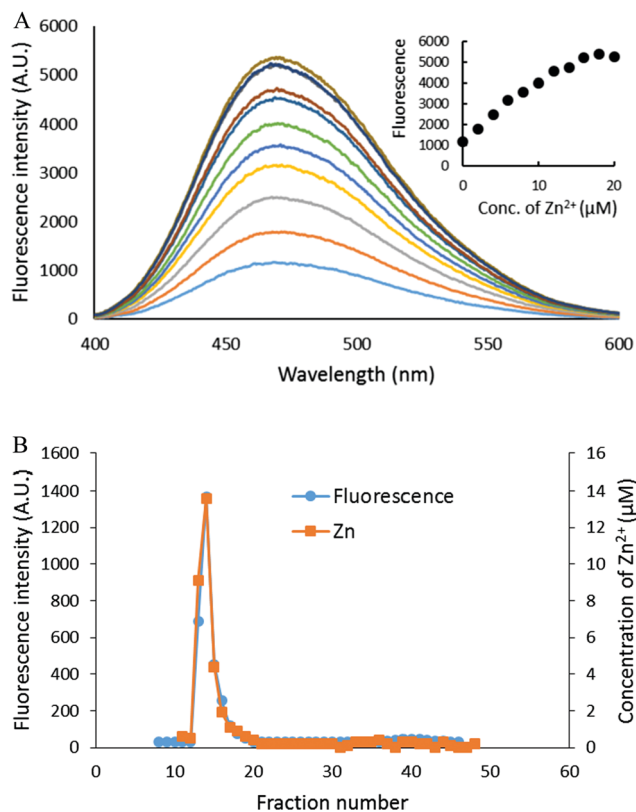


Fig. 7 Zn²⁺ titration of proteome pre-treated with ZQacid. (A) Isolated proteome (8 μM Zn²⁺) was reacted with 20 μM ZQacid for 30 min (the bottom most spectrum). Then, the reaction mixture was titrated with the increasing concentration of ZnCl₂. (B) The final reaction mixture from (A) was fractionated using Sephadex G-75 gel filtration, and the eluted fractions were analyzed for both fluorescence and zinc content.

As the number of adventitious binding sites for Zn²⁺ declines through reaction with NO, the formation of a substantial concentration of Zn(ZQ)₂ becomes more favorable (reaction (8)).

Reaction of isolated proteome with DEA-NO in the presence of FluoZin-3

The reaction of isolated proteome (10 μM Zn²⁺) with 500 μM DEA-NO was also monitored using another Zn²⁺ sensor that does not form ternary complexes with Zn-proteins, FZ-3 (20 μM). The absence of any fluorescence signal during the reaction of proteome and FluoZin-3 for 20 min indicated that Zn-proteome did not react with FZ-3 to form ternary adducts. The introduction of DEA-NO to the reaction mixture of proteome and FZ-3 for 1 hour resulted in the reduction of proteomic sulfhydryl concentration by about 41% (from 342 μM to 202 μM). The presence of DEA-NO caused a gradual increase of fluorescence throughout this period (Fig. 9A and B). Interestingly, addition of 10 μM TPEN, a powerful Zn²⁺ chelator, at an intermediate time did not quench any of the enhanced fluorescence. Instead, the fluorescence signal continued increasing. This finding suggested that the increase of fluorescence was not caused by the formation of Zn-FluoZin-3 complex, because its fluorescence should have been quenched upon the addition of TPEN. In a separate experiment, upon fractionation of the final reaction mixture of proteome (10 μM Zn²⁺), 20 μM FZ-3 and 500 μM DEA-NO using a



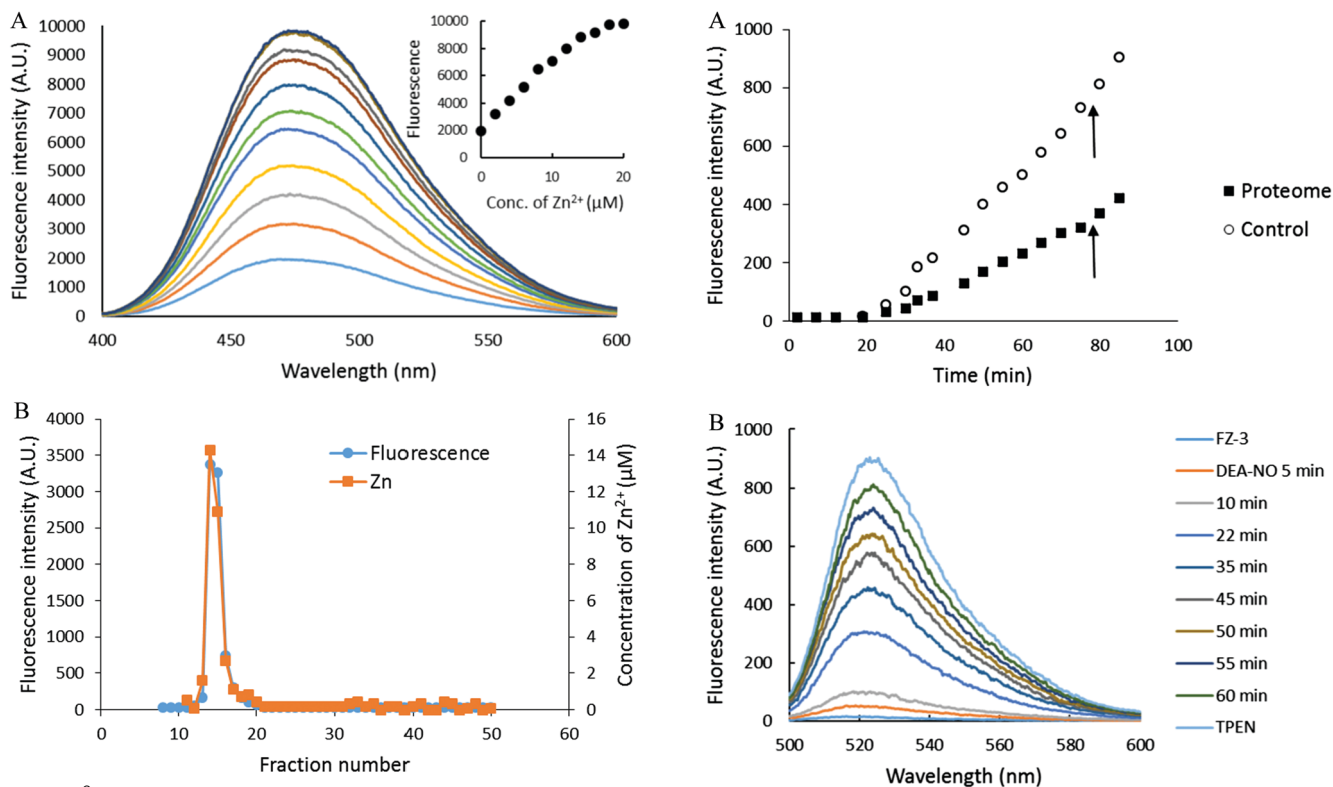


Fig. 8 Zn^{2+} titration of isolated proteome pre-treated with TSQ. (A) Isolated proteome ($8 \mu\text{M Zn}^{2+}$) was reacted with $20 \mu\text{M TSQ}$ for 30 min (the bottom most spectrum). Following on, the reaction mixture was titrated with increasing concentrations of ZnCl_2 . The inset summarizes the fluorescence changes as a function of Zn^{2+} . (B) The final reaction mixture from (A) was fractionated using Sephadex G-75 gel filtration, and the eluted fractions were analyzed for both fluorescence and zinc content.

Sephadex G-75 column, no LMW Zn-FZ-3 complex was isolated (Fig. 9C) confirming that FluoZin-3 does not chelate proteomic Zn^{2+} after reaction of DEA-NO with the proteome. However, virtually all of the fluorescence was observed in the LMW region of the chromatogram, indicating that a FZ-3 related species was responsible for the fluorescence enhancement seen during the reaction.

To investigate the source of this fluorescence enhancement, $20 \mu\text{M FluoZin-3}$, itself, was reacted with $500 \mu\text{M DEA-NO}$ for 1 hour followed by $10 \mu\text{M TPEN}$ for another 10 min (Fig. 9A). A gradual increase of fluorescence larger than observed in the proteome experiment was recorded. Again, $10 \mu\text{M TPEN}$ did not reverse the intensification of the fluorescence. It was concluded that NO reacts with FZ-3 with an increase of fluorescence, independent of its reaction with Zn^{2+} . FluoZin-3 was also reacted with *S*-nitrosylpenicillamine under similar conditions except that the reactants were kept in the dark to prevent the photochemical cleavage of S-NO bond to release NO. No reaction with FluoZin-3 was observed, indicating that the reaction of NO donors with FZ-3 is specific for nitric oxide or its oxidation products.

Discussion

Fluorescent sensors of all kinds are in routine use to reveal structures, chemical changes, and ongoing processes in living

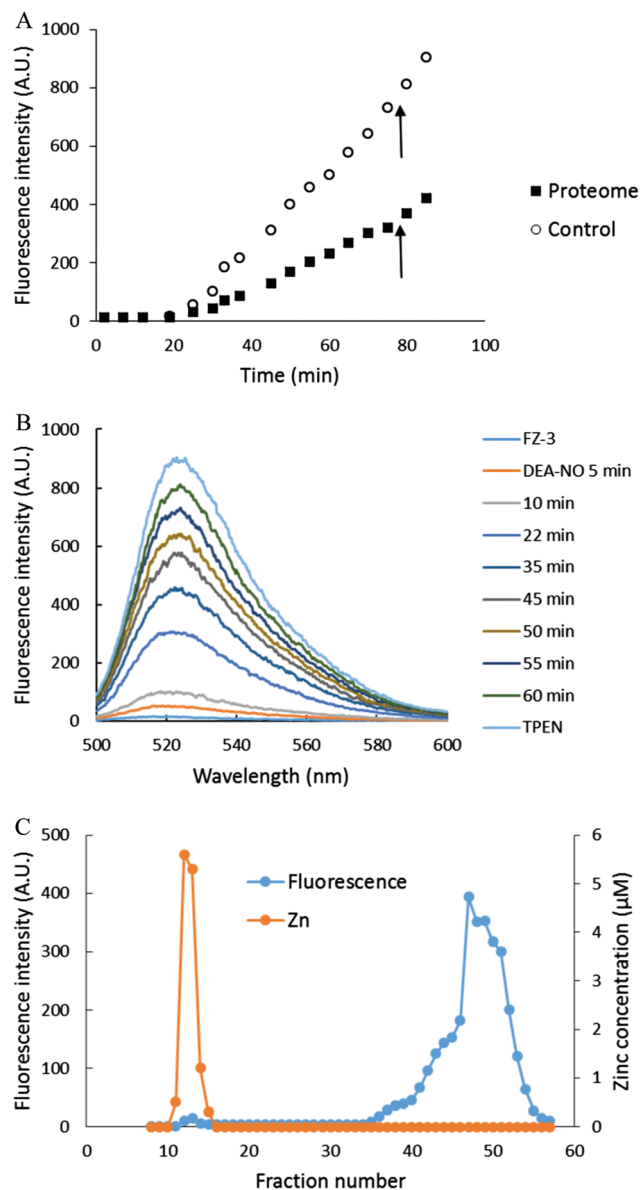


Fig. 9 Reaction of DEA-NO with isolated proteome preincubated with FluoZin-3. (A) Isolated proteome ($10 \mu\text{M Zn}^{2+}$) was reacted with $20 \mu\text{M FZ-3}$ for 20 min followed by $500 \mu\text{M DEA-NO}$ for another hour. Finally $10 \mu\text{M TPEN}$ was added for 10 min. In the control experiment, $20 \mu\text{M FZ-3}$ in 20 mM Tris (pH 7.4) was reacted with $500 \mu\text{M DEA-NO}$ for an hour followed by $10 \mu\text{M TPEN}$ for another 10 min. The arrow indicates the time of TPEN addition. (B) Fluorescence spectra of the reaction between $20 \mu\text{M FZ-3}$ and $500 \mu\text{M DEA-NO}$. (C) Sephadex G-75 chromatographic fractionation of the reaction mixture of proteome ($10 \mu\text{M Zn}^{2+}$) and $500 \mu\text{M DEA-NO}$ in the presence of $20 \mu\text{M FZ-3}$ for an hour.

cells.^{28–31} Major efforts have been made and are continuing to design probes that selectively detect essential and extraneous metal ions in cells and, thereby, to reveal their participation in normal and pathologic biochemistry.^{1,3,30,32,33} In the case of Zn^{2+} , for example, the appearance of a fluorescence microscopic signal in the presence of a cell permeable sensor, followed by its diminution in the presence of the powerful membrane permeant chelator, TPEN, has been employed in many studies to conclude



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