Studying metal-protein interactions using fluorescent protein indicators

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Abstract

Metals are widespread environmental toxins that disrupt normal cellular processes through their interactions with proteins and other macromolecules. In this study, we developed the metal-sensitive fluorescent protein msGFP as a ratiometric reporter capable of binding heavy metals, and adapted the redox reporter roGFP2 as a ratiometric indicator that binds transition metals. As ratiometric reporters, we found that msGFP bound mercury and lead tightly but had substantially lower affinity for other metals, whereas roGFP2 bound copper tightly, with weaker affinity for nickel and cobalt. Furthermore, msGFP and roGFP2 could report metal binding through fluorescence quenching, and we used this effect to measure high affinity interactions for both proteins with copper and ferrous iron. Crystal structure analysis of msGFP complexed with phenylarsine oxide revealed an unexpected mode of heavy metal interaction, with msGFP binding PAO with 2:2 stoichiometry. Glutathione strongly inhibited most metal interactions with the fluorescent protein reporters, but increased the affinity of arsenic and cadmium for msGFP in a dose-dependent fashion. When expressed in HEK293T cells, msGFP reported uptake of mercury and phenylarsine oxide from surrounding media. We also found that glutathione depletion enhanced binding of phenylarsine oxide to msGFP, validating the importance of glutathione in modulating metal-protein interactions.

Introduction

Metals are widespread and persistent environmental toxins with profound impacts on public health. Given their abundance and potency, heavy metals such as arsenic, lead, mercury, and cadmium are considered among the most hazardous toxins in the lived environment(1,2). Toxic exposure to heavy metals affects more than one hundred million people globally(3) and is linked to a broad spectrum of illnesses, including cardiovascular disease(4), cognitive disorders(5), and cancer(6,7). Even less toxic metals, including essential micronutrients such as copper, iron, and zinc can also produce adverse effects from excessive exposure or diseases that disrupt homeostasis(8).Having reporters for these metals is vital for understanding how metals interact with cellular factors and for studying processes that affect metal uptake and homeostasis(9).

Toxic metal exposure harms cells through various mechanisms, including by producing reactive oxygen species and disrupting enzyme function(10). For example, some metals can generate free radicals through catalysis of peroxide(11,12) or by activating oxidizing enzymes(13). Metals can also exhaust cellular antioxidants by inhibiting redox enzymes such as thiol oxidoreductases(14,15). Generally, metals can disrupt normal enzyme functions involved in a wide range of physiological processes, including heme biosynthesis, oxidative phosphorylation, and DNA repair(2). Heavy metals are particularly disruptive to enzymes with cysteine-containing motifs as heavy metals have a strong affinity for thiols(16).

Metals can also interact with abundant cellular small molecules that have metal coordination properties. These low molecular weight ligands alter metal interactions with proteins and other cellular factors by catalyzing ligand exchange(17,18) or through the formation of ternary complexes that alter their mode of binding(19). Glutathione is one of the most abundant low molecular weight ligands, with millimolar cellular concentrations(20). Reduced glutathione

(GSH) can form complexes with transition and heavy metals, consequently altering their interactions inside the cell. Glutathione is protective against metal stress(21,22), and this effect is generally attributed to its role as a cellular antioxidant. However, glutathione complexation may also provide direct protection from heavy metals by facilitating export(23). Despite the abundance of glutathione in cells, its effect on metal-protein interactions has only been examined in select cases(17).

We used fluorescent proteins to measure metal interactions with proteins featuring surface proximal cysteines. These include the reduction-oxidation-sensitive GFP (roGFP2) previously developed to measure cellular redox levels(24,25) and a related metal-sensitive GFP (msGFP) explicitly designed for this study. We report metal interactions with both proteins *in vitro* and with msGFP in mammalian cells. Finally, we determined the influence of GSH on these interactions, demonstrating that physiological concentrations of glutathione dramatically alter metal interactions with proteins.

Results

We created a fluorescent metal reporter, metal-sensitive GFP (msGFP), based on the design of the redox-sensitive fluorescent protein roGFP2(24,25). roGFP2 is a redox reporter protein with proximal cysteines (C147, C204) placed with a Cα distance of 4.4-4.8Å. Under oxidizing conditions, the formation of a strained disulfide bond causes a conformational change that alters the fluorescence properties of roGFP2(25). We used a cysteine pair (C147, C202) in msGFP with a Cα distance of 7.3Å, far enough apart to preclude the formation of disulfide bonds, but still close enough to allow for a similar conformational change resulting from metal binding (Fig. 1A).

We performed titrations with msGFP and metals with known thiol-binding characteristics. We first determined that Hg^{2+} induced a ratiometric change in the excitation spectra of msGFP (Fig. 1B) similar to the effect produced by oxidation on roGFP2(24). All tested heavy metals (Pb^{2+}, Hg^{2+} Cd^{2+}, As^{3+}) induced observable changes in ratiometric msGFP fluorescence, with Pb^{2+} (Kd = 2.6 μM) and Hg^{2+} (Kd = 1.0 μM) having the highest potency. Cd^{2+} (Kd = 640 μM) and As^{3+} (Kd > 10 mM) had a more modest affinity as measured by change in msGFP fluorescence (Fig. 1C). For each of these metals except As^{3+}, fluorescence changes were reversed upon addition of 5 mM EDTA (Fig. 1D). By comparison, transition metals (Fe^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, Cu^{2+}) did not produce a ratiometric change in msGFP fluorescence. The post-transition metal Zn^{2+} produced a change in fluorescence comparable to that induced by As^{3+} (Fig. S1A).

In titrations with metals and roGFP2, the transition metals Cu^{2+} (Kd = 0.3 μM), Ni^{2+} (Kd = 5.9 μM), and Co^{2+} (Kd = 55.4 μM) produced changes in ratiometric fluorescence (Fig. 1E), whereas heavy metals did not (Fig. S1B). The transition metal-induced changes in ratiometric roGFP2 fluorescence were reversible by EDTA and not β-mercaptoethanol (Fig. 1F, S2), indicating that this effect is caused directly by metal binding and not by disulfide formation(26,27). Addition of H_2O_2 induced a ratiometric change in roGFP2 fluorescence at high concentrations, consistent with previous results(24), but had no effect on msGFP fluorescence (Fig. S1C).

We observed fluorescence quenching for both msGFP and roGFP as metals were added. This effect was particularly noticeable with transition metals, which form complexes that absorb light in the visible spectrum. We hypothesized that this was due to quenching by FRET with the GFP chromophore, previously observed in fluorescent proteins with engineered metal binding sites(28,29). FRET is a distance-dependent, non-radiative form of energy transfer whose efficiency also depends on the overlap between the emission of the donor fluorophore and the

absorbance of the acceptor chromophore(30). We observed the strongest quenching with transition metals (Cu^{2+}, Fe^{2+}, and Fe^{3+}) whose absorbance spectra overlapped with the identical emission spectra of roGFP2 and msGFP (Fig. 2A, left). FRET radius (R_0) values calculated for these fluorescent proteins with Cu^{2+} (13.3Å), Fe^{3+} (15.1Å), and Fe^{2+} (9.1Å) support the possibility of FRET-dependent quenching, as these distances exceed the GFP chromophore-C147 side chain distance of 7.5Å in roGFP2(25) (Fig. 2A, right). Quenching for msGFP (excited at 488 nm) clearly followed the expected pattern for single site binding (Fig. 2B), with Cu^{2+} (Kd = 0.5 μM) and Fe^{3+} (Kd = 1.2 μM) binding more tightly than Fe^{2+} (Kd = 120 μM). For Cu^{2+} and Fe^{3+}, quenching was abolished by the inclusion of 5 mM EDTA in the assay buffer, signifying that quenching was the result of direct interaction between these metals and msGFP. Some quenching was still observed for Fe^{2+} even in the presence of excess EDTA, with this possibly caused by the inner filter effect(31). In titrations with roGFP2, we observed similar fluorescence quenching curves for Fe^{3+} (Kd = 2.2 μM) and Fe^{2+} (Kd = 120 μM) as with msGFP (Fig. 2C). However, Cu^{2+} appeared to quench roGFP2 with lower affinity (Kd = 8.0 μM) compared to msGFP, although the binding model does not account for ratiometric changes in roGFP2 fluorescence (Fig. 1D).

We observed that As^{3+} bond to msGFP with weak affinity (Fig. 1A), although it had been previously reported to bind similar sites more tightly(32). We sought to determine how arsenic speciation might affect this interaction. Inorganic arsenic has two valence states, with trivalent As^{3+} interacting with thiol groups in proteins(33). Beyond that, a diverse assortment of organoarsenical compounds are also found in the environment, having been formed through biological or chemical synthesis(34). Consistent with previous studies, the pentavalent As^{5+} did not bind msGFP (Fig. 3). However, the organoarsenical compound phenylarsine oxide (PAO) bound msGFP with an affinity (Kd = 2.4 μM) comparable to that of Pb^{2+} and Hg^{2+} (Fig. 1B) and exceeding the affinity for As^{3+} by nearly four orders of magnitude. These data suggest that substitution might increase the affinity of As^{3+} for protein thiols (Fig. 3B).

We next evaluated the effect of glutathione on metal-msGFP interactions. Glutathione is the central redox agent and most abundant thiol in most aerobic organisms(35). In mammalian cells, glutathione concentrations range from 1-10 mM(20), with the reduced form comprising ~98% of the cellular pool(36). Including 1 mM reduced glutathione (GSH) included in the assay dramatically altered the metal-msGFP affinity of every tested metal (Fig. 4). We observed a significant increase in the affinity of As^{3+} for msGFP (Fig. 4A, left) likely resulting from the formation of labile $As(GS)_3$ complexes that readily react with proteins(37,38) (Fig. 4A, right). Overall, we observed significant increases in msGFP binding affinity for As^{3+} and Cd^{2+} and a modest increase for Zn^{2+}. By comparison, we saw decreases in affinity for all other metals tested (Fig. 4B). When included in titrations with roGFP2, 1 mM glutathione decreased the affinity of all metals except for Co^{2+}, which was not affected (Fig. 4C). For both proteins, the effect of glutathione on metal binding was usually substantial, often changing affinities by an order of magnitude or more (Fig. 4B, C). We also observed that the effect of glutathione was concentration-dependent, with various concentrations within the range of physiological variation having distinct effects on metal binding. For As^{3+} and GSH into msGFP revealed a GSH-dependent increase in As^{3+} binding at GSH concentrations up to 3 mM, followed by a decrease at 10 mM GSH (Fig. 4D). By comparison, any addition of GSH reduced the affinity of PAO for msGFP (Fig. 4D). Titrations for Cd^{2+} and Zn^{2+} with GSH also revealed GSH-dependent affinity increased for msGFP, although Cd^{2+} precipitated with higher concentrations of GSH(39) (Fig. 4D).

Next, we determined the efficacy of msGFP in reporting metal binding within cells. Surprisingly, exposing HEK293T cells stably expressing msGFP to micromolar concentrations of metals

largely had no effect on msGFP fluorescence (Fig. 5A). However, Hg^{2+} and PAO induced ratiometric changes in cellular msGFP fluorescence consistent with results obtained *in vitro* (Fig. 3B). We found that PAO-induced changes in msGFP fluorescence occurred more rapidly than observed for Hg^{2+}, likely due its increased cell permeability. However, we found that the effect of Hg^{2+} was stronger (Fig. 5A). We then determined the effect of glutathione depletion on msGFP binding with PAO, interactions for which glutathione had large effects *in vitro* (Fig. 4B). In msGFP-expressing HEK293T cells pretreated with the glutamate-cysteine ligase inhibitor(40) buthionine sulfoximine (BSO, 100 μM), we found an increased rate of PAO-induced fluorescence change (Fig. 5B).

We also attempted structural determination of msGFP-metal complexes with crystallization screens of msGFP and interacting metals. Among these, msGFP-PAO produced crystals that diffracted to 1.89Å. Following molecular replacement and refinement, we produced a crystallographic model that surprisingly placed PAO at the interface between subunits of an antiparallel msGFP dimer in a 2:2 conformation, linking C147 of one subunit to C202 of the other (Fig. 6A). Our composite OMIT (2mFo-DFc) map(41) supported the placement of PAO at this position (Fig. 6B). In contrast to previous studies comparing the structural arrangement of oxidized (1JC1) and reduced (1JC0) forms of roGFP2(25), we were unable to identify any significant structural changes in PAO-bound msGFP compared to reference eGFP(42) (Fig. 6C).

Discussion

In this study, we engineered the fluorescent protein msGFP from roGFP2, and compared the ability of both fluorophores to report interactions between metals and proteins. We noted that roGFP2 exhibited a previously unreported sensitivity to transition metals, with a reversible change in ratiometric fluorescence caused by titrations of Ni^{2+}, Co^{2+}, and Cu^{2+} that fit a single site binding model (Fig. 1B). Cu^{2+}, but not Ni^{2+} or Co^{2+}, also bound msGFP as determined by fluorescence quenching (Fig. 2B). Interestingly, heavy metals induced a measurable ratiometric change in msGFP, but had no observable effect on roGFP2 (Fig. 1, S1). Since msGFP differs from roGFP2 only in the distance between its metal-binding cysteine residues (Fig. 1A), these variations in metal ion selectivity may be explained by differences in bond lengths and coordination geometries(43,44). This potentially also explains the discrete Bmax values observed for each metal (Fig. 1B, 1C).

We observed fluorescence quenching in both reporters for several of the metals tested. The strongest quenching was observed for the colored transition metals Cu^{2+}, Fe^{2+}, and Fe^{3+}, whose absorbance overlaps with the identical emission spectra of msGFP and roGFP2 (Fig. 2A). The spectral properties of Cu^{2+} have been exploited in measuring short-range interactions by FRET(45-47). FRET with an iron acceptor has similarly been observed in experiments linking a heme protein with eGFP(48). One surprising finding in our studies was that Cu^{2+} had a greater quenching effect on msGFP compared to roGFP2 (calculated transfer efficiency of 0.94 for msGFP compared to 0.80 for roGFP2) (Fig. 2), even though one of the coordinating cysteines (C202 for msGFP, C204 for roGFP2) is located further from the GFP chromophore. Transition metals lack the orientation dependence of organic chromophores for FRET(47) and quenching efficiency should depend entirely on the distance between the donor fluorophore and the acceptor Cu^{2+} ion. The fact that msGFP and roGFP2 do not follow this pattern raises the possibility that other quenching processes may be involved in Cu^{2+}-mediated quenching of msGFP.

Cells contain pools of small molecules that modulate metal interactions with proteins(17). Among the most abundant of these is glutathione, which in its reduced form is the predominant

thiol in aerobic organisms(20,35). We found that GSH inhibited most interactions between metals with either msGFP or roGFP2 (Fig. 4). However, GSH increased the affinity of As^{3+} and Cd^{2+} for msGFP, likely through the formation of ternary complexes(39). In the presence of excess glutathione, arsenite forms $As(GS)_3$ complexes that subsequently bind proteins through thiol exchange(37,38). We found that GSH enhances As^{3+} binding to msGFP in a concentration-dependent fashion up to 3 mM, with higher concentrations of GSH exerting an inhibitory effect (Fig. 4D). By contrast, GSH at any concentration inhibited the interaction between the organoarsenical PAO and msGFP. These findings suggest that, as with other metals, GSH also acts as a competitive inhibitor of arsenic-protein interactions. This is also likely true for Cd^{2+}, which showed a similar GSH-dependent increase in affinity as concentrations exceeded 100µM (Fig. 4D). We found that binding of Zn^{2+} to msGFP was enhanced by addition of GSH, although this effect was more modest than those observed for As^{3+} and Cd^{2+}. Our findings are in line with previous observations that GSH alters the kinetics of Zn^{2+} binding to fluorescent indicators(19,49).

A surprising finding in our study is the arsenic selectivity of msGFP expressed in HEK293T cells. Metals that strongly bound msGFP *in vitro*, such as Hg^{2+} and Pb^{2+}, had almost no effect on cellular msGFP fluorescence (Fig. 6A). By comparison, PAO had a rapid and profound effect on msGFP fluorescence (Fig. 6A). As^{3+} also affected the cellular fluorescence of msGFP, although these effects were slower and less profound compared to those induced by the same concentration of PAO (Fig. 6A). It is important to note that unlike other heavy metals, arsenic almost exclusively bonds to cellular thiols(33). We found that depleting glutathione by pretreatment with BSO had effects in agreement with our *in vitro* assays with these metals (Fig. 4D). As expected, pretreatment with BSO reduced As^{3+} and increased PAO-induced changes in msGFP fluorescence (Fig. 6B). Collectively, we interpret these results as indicating that other factors, such as transporters, metallothioneins, and other low molecular weight ligands play a determinative role in metal-protein interactions in cells.

Experimental procedures

Reagents

Copper (II) chloride dihydrate (Cu^{2+}), cobalt (II) chloride hexahydrate (Co^{2+}), iron (II) sulfate heptahydrate (Fe^{2+}), iron (III) chloride (Fe^{3+}), nickel (II) chloride hexahydrate (Ni^{2+}), zinc (II) sulfate heptahydrate (Zn^{2+}), cadmium (II) chloride hemipentahydrate (Cd^{2+}), lead (II) nitrate (Pb^{2+}), mercury (II) chloride (Hg^{2+}), and hydrogen peroxide (H_2O_2, 30% in water) were purchased from Fisher Scientific. Buthionine sulfoximine (BSO), sodium arsenate (V) (AsO_4^{3-}, herein described as As^{5+}), sodium arsenite (III) (AsO_3^{3-}, herein described as As^{3+}), and phenylarsine oxide (PAO) were purchased from Millipore Sigma. Except for phenylarsine oxide, metal stocks were prepared at 100 mM in water, with the pH adjusted to 7 with 1M NaOH. Phenylarsine oxide stocks were prepared in DMSO.

Plasmid constructs

pQE-60 roGFP2-His was a gift from Tobias Dick (Addgene #65046)(50). The construct for msGFP was generated by substitution of codons encoding two amino acids (S202C, C204C) from parent roGFP2. Both msGFP and roGFP2 were cloned into the bacterial expression plasmid pKM260. msGFP was also cloned into pcDNA3 (Invitrogen) for mammalian expression. All cloning was performed by NEB HiFi assembly (New England Biolabs) and validated by whole plasmid sequencing (Plasmidsaurus).

Protein expression and purification

pKM260-msGFP and pKM260-roGFP2 were transformed into BL21(DE3)-pRARE2 cells by electroporation (0.1 mM gap, 2,000 V) at 4˚C and plated on LB plates containing 100 µg/mL of ampicillin. Transformed cells were grown in 2XYT media containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol, with protein expression induced by autoinduction(51) during growth at 23˚C for 24 hours.

Following expression, cells were lysed by ultrasonication (500 W, 30% duty cycle, 2 seconds on, 10 seconds off for 2 minutes) in cold start buffer (20 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 8) and clarified by centrifugation (28000 x G for 30 minutes at 4˚C). Both msGFP and roGFP2 were purified by NiNTA chromatography (QIAGEN) over a column bed containing 5ml of resin equilibrated with start buffer. The bound proteins were washed three times in wash buffer (20 mM Tris, 150 mM NaCl, 30 mM imidazole, pH 8) followed by elution in elute buffer (20 mM Tris, 150 mM NaCl, 200 mM imidazole pH 8). The N-terminal polyhistidine tag was removed by overnight dialysis (20 mM Tris, 150 mM NaCl, 1 mM beta-mercaptoethanol) with TEV protease at 4˚C. The dialyzed protein was again purified by NiNTA chromatography, collecting the flow through. This was then diluted by the addition of two volumes of salt-free buffer (20 mM Tris, 1 mM beta-mercaptoethanol, pH 8), loaded onto a HiTrap Q HP column (Cytiva) equilibrated with 20 mM Tris, pH 8, and eluted using a gradient from 0.05-1M NaCl. Fractions containing proteins were concentrated by ultrafiltration and purified by size exclusion on a Sephacryl S200 column (Cytiva) in SEC buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7). The proteins typically eluted in two peaks, with fractions from the later peak used for our experiments.

Determination of msGFP and roGFP2 fluorescence properties

We measured the absorbance and fluorescence emission spectra of msGFP and roGFP2 in the same buffer used for SEC (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7). For absorption, we used a BioSpectrometer (Eppendorf). Fluorescence excitation-emission spectra were collected using a Fluoromax-3 (HORIBA) fluorometer. Absolute concentrations were determined using the absorption of the chromophore in 0.1 M NaOH, which for GFP has an extinction coefficient of 44000 $M^{-1}cm^{-1}$ (52). The results of our experiments revealed quantum yield and emission spectra for msGFP and reduced roGFP2 that were indistinguishable from those of eGFP(53).

Calculation of FRET radii for transition metals

FRET efficiency depends on the distance between the donor and acceptor molecule and the Förster radius (R_0) as described by the following formula:

$$E = \frac{1}{1+(r/R_0)^6}$$

We calculated R_0 (the distance at which FRET efficiency is 50%) using established methods(31) and assuming an orientation factor κ^2 of 2/3. We estimated the refractive index n as 1.3348, the value described for PBS(54). For determination of spectral overlap with GFP fluorophores, we measured the absorbance of metal complexes using a BioSpectrometer (Eppendorf). When taking these measurements, we attempted to determine the absorbance of metals in complex with thiol-based chelators such as dimercaptosuccinic acid (DMSA) and free cysteine. However, these readings were unstable, and we used EDTA complexes as an approximation instead.

Fluorescence plate reader assays

For fluorescence assays, 200 µL reactions were set up in individual wells of black 96-well microplates (BRAND) containing 30 nM of fluorescent protein (roGFP2 or msGFP) and the indicated dilution of each metal or hydrogen peroxide. Titrations were performed in reducing assay buffer (20 mM HEPES, 150 mM NaCl pH 7). We observed that millimolar quantities of reducing agents formed insoluble complexes with some metals; consequently, all protein stocks (which contain TCEP) were diluted >1000 fold for each assay. Experiments involving glutathione included the indicated amount of reduced glutathione, freshly prepared as a 1M stock in water and equilibrated to pH 7 with sodium hydroxide. All reactions were allowed to equilibrate for 30 minutes at room temperature. Readings were performed on a Cytation 5 (BioTek) multimodal plate reader at room temperature. Monochromators were set to either 400 or 485 nm with slit widths of 20 nm for excitation, and 528 nm with a slit width of 20 nm for emission. Samples exhibiting visible precipitation or nonspecific quenching due to light absorbance were excluded from analysis. Binding for samples excepting Fe^{2+} and Fe^{3+} was measured by a ratiometric change in fluorescence indicated by the formula $F(\lambda_{x400}, \lambda_{m528}) / F(\lambda_{x485}, \lambda_{m528})$. Binding for Fe^{2+} and Fe^{3+} was measured by the total change in $F(\lambda_{x488}, \lambda_{m528})$ as a result of quenching. Binding for Cu^{2+} was evaluated by ratiometric fluorescence change for roGFP2 and by quenching for msGFP. Binding data was averaged across experimental days and protein preparations and fitted with a total binding model using Prism 9 (Graphpad).

Crystallography

Initial crystal screening was performed using the Crystal Screen HT (Hampton) with 20 mg/ml msGFP in SEC buffer (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7). Screens were also performed with msGFP supplemented with 1 mM As^{3+}, Pb^{2+}, Cd^{2+}, phenylarsine oxide (PAO), or 1 mM As^{3+} supplemented with 3 mM glutathione. After optimization, only msGFP supplemented with PAO produced diffracting crystals. Crystals grown in 0.2 M Li_2SO_4, 0.1 M Tris pH 8, 32% PEG 3350 were used for cryopreservation and data collection. X-ray diffraction data was collected on a Rigaku R-Axis equipped with HTC plate reader (Rigaku) and processed using HKL2000 (HKL Research). Crystals conformed to the cubic space group I23. Phases were solved by molecular replacement using PHASER-MR(55) with roGFP2 (PDB 1JC0) as the template. Model refinement and the generation of an iterative build OMIT map(41) were performed using Phenix(56) and Coot(57).

Cell imaging assays

HEK293T cells were cultured in standard culture media (DMEM, 10% FBS, 1X penicillin-streptomycin) (Gibco). Cells grown to >90% confluence in 35 mm plates were transfected with pcDNA3-msGFP linearized with MluI using Lipofectamine 2000 (Thermo) according to the manufacturer's instructions. One day post-transfection, cells were removed by treatment with trypsin (0.05%) and plated at 1:10 dilutions in the wells of a 6-well plate containing culture media with dilutions of G418 at concentrations ranging from 0-500 µg/mL. Cells were evaluated for fluorescent colony formation at periodic intervals up to 10 days post transfection. Discrete colonies were selected from wells with G418 concentrations of 300 µg/mL. Colonies were isolated following brief treatment by trypsin and expanded in culture media containing 200 µg/mL G418. For assays involving glutathione depletion, buthionine sulfoximine (Sigma) was added to achieve a final concentration of 100 µM and incubated for 16 hours before data collection.

For imaging experiments, stably transfected cells were incubated in HBSS (Gibco) before adding the indicated amount of metal. Fluorescence imaging was collected over a time using a Nikon A1R inverted microscope with 20X objective and excitation wavelengths of 405 nm (60 power, 125 gain) and 488 nm (11 power, 65 gain) paired with emissions recorded between 520-600 nm and -1 PMT offset. Images were captured using a Galvano scanner, and ratiometric images were collected and analyzed using NIS Elements (Nikon).

Data availability

Research data is included as supporting information in this manuscript. The structural model of msGFP-PAO is deposited with the Protein Data Bank (PDB 8DTA).

Supporting information

This article contains supporting information, including raw data and supplementary figures.

Funding and additional information

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

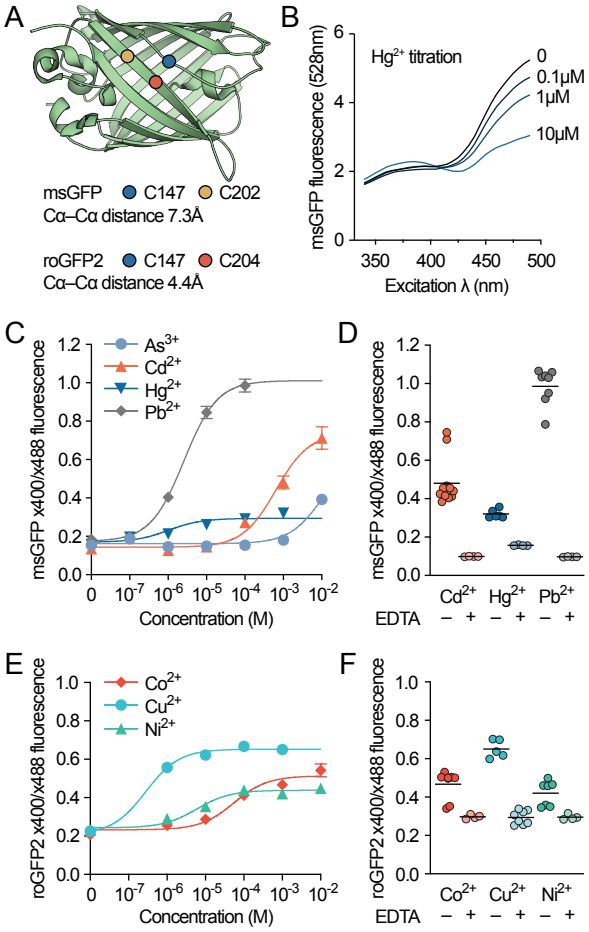

Figure 1. Measuring metal-protein interactions with fluorescent reporters. *A*, engineered cysteine sites for msGFP and roGFP2 mapped on reference GFP structure (PDB 4EUL). *B*, excitation spectra of msGFP during titration of Hg^{2+}. Left axis indicates relative fluorescence of emission measured at 528nm. *C*, ratiometric change in msGFP fluorescence during titrations of indicated metals. Error bars indicate SEM. *D*, reversal of metal-induced (1 mM for Cd^{2+} and 100μM for Pb^{2+} and Hg^{2+}) ratiometric fluorescence changes in msGFP by addition of 5 mM EDTA. *E*, same as (*C*), but for roGFP2. *F*, reversal of metal-induced (1 mM for all metals shown) fluorescence changes in roGFP2 by addition of 5 mM EDTA.

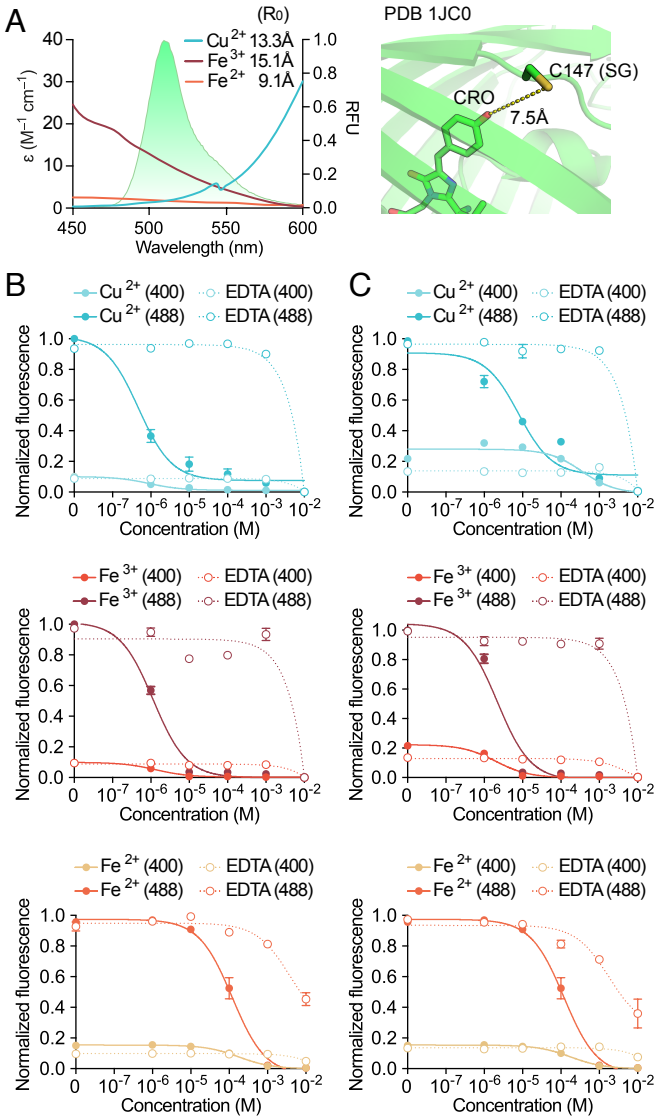

Figure 2. msGFP and roGFP2 quenching by metal chromophores. *A*, overlay of FP emission spectra (identical between msGFP and roGFP2) with absorption spectra of coordinated Cu^{2+}, Fe^{3+}, and Fe^{2+} complexes. Also shown (inset) is the calculated Forster radii (R_0), and (right) the distance between the phenolate of the GFP chromophore and the thiol of C147 in roGFP2 (PDB 1JC0). *B*, quenching of msGFP fluorescence with indicated metals in (*A*) along with its reversibility by inclusion of 5mM EDTA. Error bars indicate SEM. *C*, same as (*B*) but for roGFP2.

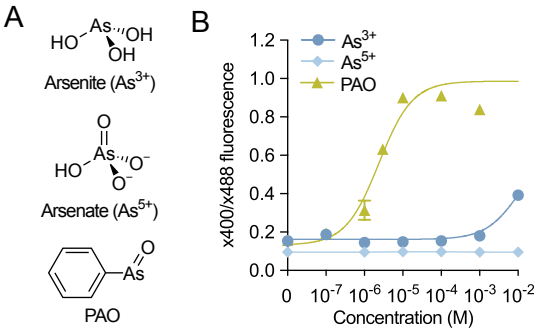

Figure 3. Arsenic speciation affects reactivity with msGFP. *A*, ionic form of arsenic species used in this assay (pH 7). As^{3+} data reproduced from Fig 1. *B*, ratiometric change in msGFP fluorescence during titrations of indicated arsenic species. Error bars indicate SEM.

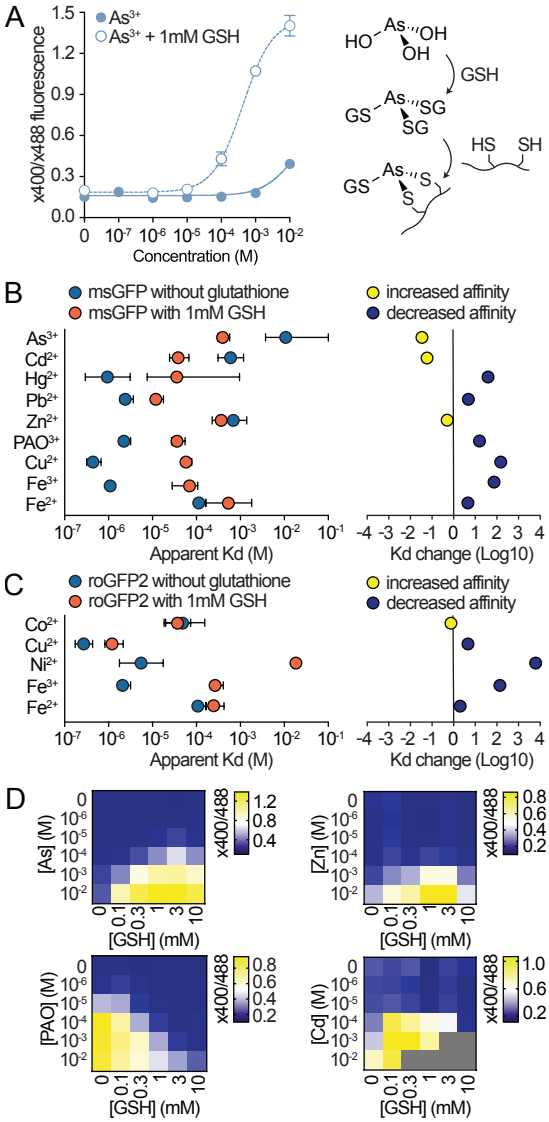

Figure 4. Glutathione alters interactions between metals and fluorescent reporters. *A*, ratiometric fluorescence of msGFP in a titration of As^{3+} with or without 1 mM glutathione. SEM indicated by error bars. Hypothesized mechanism (right) of glutathione mediated As^{3+} interaction with cysteine pairs. *B*, Summarized effects of 1mM glutathione on the interactions of msGFP with indicated metals. Error bars indicate the confidence intervals of Kd estimates. Affinity changes (right) between msGFP and indicated metals after addition of glutathione. *C*, Same as for (*B*), but with roGFP2. D, msGFP titrations with As^{3+}, Zn^{2+}, PAO, and Cd^{2+} against glutathione. Scale for ratiometric titration is shown (right) for each array. Grey boxes indicate samples that could not be acquired due to precipitation.

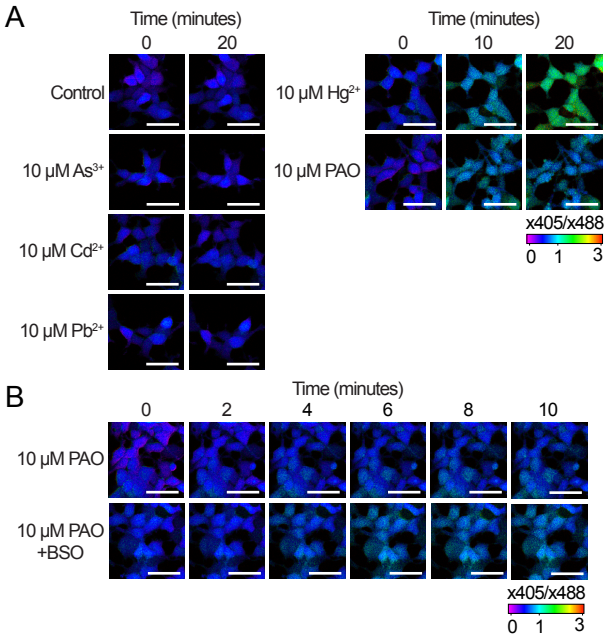

Figure 5. Heavy metals alter msGFP fluorescence in HEK293T cells. *A*, ratiometric fluorescence of msGFP in HEK293T cells after exposure to heavy metals. Expanded timecourses (at right) shown for metals that produced changes in msGFP fluorescence. 50 μm scale bars are shown for reference. *B*, glutathione depletion with BSO (100 μM) increased the rate of PAO-induced effects on msGFP fluorescence in HEK293T cells. 50 μm scale bars are shown for reference.

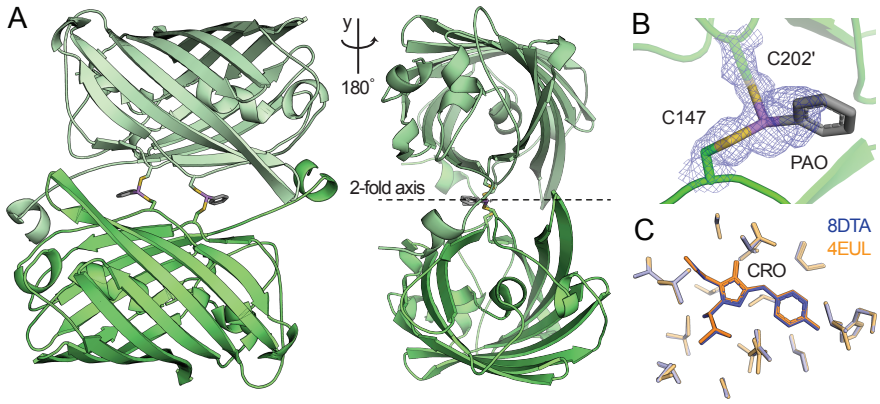

Figure 6. Crystallographic structural model of msGFP-PAO complexes. *A*, model of msGFP-PAO complexes resolved by x-ray crystallography. An msGFP monomer (dark green) complexes with PAO in a 2:2 stoichiometry with its symmetry mate (light green). Rotated view (right) of the msGFP-PAO complex showing the 2-fold rotation axis. *B*, composite OMIT map of one PAO coordinated by C147 and C202 across two msGFP monomers. *C*, superposition of msGFP-PAO (PDB 8DTA) on reference GFP (PDB 4EUL) showing that the chromophore environment is largely unchanged between the two structural models.

Table 1. Data collection and refinement statistics.

Resolution range	33.89 - 1.811 (1.875 - 1.811)
Space group	I 2 3
Unit cell	126.802 126.802 126.802 90 90 90
Total reflections	542477 (53313)
Unique reflections	30947 (3068)
Multiplicity	17.5 (17.4)
Completeness (%)	99.71 (100.00)
Mean I/sigma(I)	33.07 (3.83)
Wilson B-factor	23.40
R-merge	0.1114 (1.018)
R-meas	0.1148 (1.049)
R-pim	0.02724 (0.2504)
CC1/2	0.999 (0.85)
CC*	1 (0.959)
Reflections used in refinement	30866 (3068)
Reflections used for R-free	1989 (198)
R-work	0.1620 (0.2180)
R-free	0.1813 (0.2684)
CC(work)	0.970 (0.889)
CC(free)	0.965 (0.830)
Number of non-hydrogen atoms	2172
macromolecules	1868
ligands	95
solvent	250
Protein residues	234
RMS(bonds)	0.013
RMS(angles)	1.24
Ramachandran favored (%)	99.13
Ramachandran allowed (%)	0.87
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.49
Clashscore	1.58
Average B-factor	28.98
macromolecules	27.72
ligands	35.49
solvent	36.97

Statistics for the highest-resolution shell are shown in parentheses.

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