
Design of a highly specific and noninvasive biosensor suitable for real-time in vivo imaging of mercury (II) uptake

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Abstract

Mercury is a ubiquitous pollutant that when absorbed is extremely toxic to a wide variety of biochemical processes. Mercury (II) is a strong, “invisible” poison that is rapidly absorbed by tissues of the intestinal tract, kidneys, and liver upon ingestion. In this study, a novel fluorescence-based biosensor is presented that allows for the direct monitoring of the uptake and distribution of the metal under noninvasive in vivo conditions. With the introduction of a cysteine residue at position 205, located in close proximity to the chromophore, the green fluorescent protein (GFP) from *Aequorea victoria* was converted into a highly specific biosensor for this metal ion. The mutant protein exhibits a dramatic absorbance and fluorescence change upon mercuriation at neutral pH. Absorbance and fluorescence properties with respect to the metal concentration exhibit sigmoidal binding behavior with a detection limit in the low nanomolar range. Time-resolved binding studies indicate rapid subsecond binding of the metal to the protein. The crystal structures obtained of mutant eGFP205C indicate a possible access route of the metal into the core of the protein. To our knowledge, this engineered protein is a first example of a biosensor that allows for noninvasive and real-time imaging of mercury uptake in a living cell. A major advantage is that its expression can be genetically controlled in many organisms to enable unprecedented studies of tissue specific mercury uptake.

Keywords: mercury; biosensor; GFP; fluorescence

Supplemental material: see www.proteinscience.org

Understanding the uptake and distribution of toxic metals is crucial to the diagnosis and identification of heavy metal induced diseases and contaminations. Many of these metals are hazardous when absorbed at even minute concentrations, causing severe neurobehavioral effects

and cardiovascular and digestive diseases. Mercury, in particular, is a very potent inhibitor to many protein functions as it can readily be absorbed in the lungs, through the skin, or by ingestion. Many of the diseases induced by the absorption of mercuric compounds are most frequently caused by a chronic low-level exposure to the metal or by temporary high-level exposure due to human negligence and pollution. The uptake of mercury through inhalation predominantly affects bronchial tissues, whereas mercury absorption by ingestion primarily affects stomach, intestine, kidneys, and liver. Hydrophobic organomercurials can also cross the blood-brain barrier,

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enabling its accumulation in the brain. Mercury chloride can be converted into organomercurials, such as methyl mercury, which are toxic due to strong interactions with the hydrophobic environments of proteins and membranes. Mercuric compounds are generally difficult to degrade and to dispose and thus accumulate in cells and tissues. The extreme toxicity of this metal can, in part, be explained by its strong affinity to sulfhydryl groups of many proteins, thereby altering structural and functional states.

A variety of analytical tools are commonly employed to detect mercury in biological samples. These include inductively coupled plasma mass spectrometry (Centineo et al. 2005), gas chromatography (Nevado et al. 2005), as well as flame and emission spectroscopy (Pereiro and Diaz 2002). Other methods include, for example, nuclear microscopy, X-ray fluorescence, voltametric measurements (Barek et al. 2001), and the use of colorimetric probes (Coronado et al. 2005; Ko et al. 2006). Many of these analytical tools are highly sensitive and enable accurate measurements of mercury in the low part per million and part per billion ranges (Yoon et al. 2005).

While traditional analytical detection methods allow for highly accurate measurements of metal concentrations, they commonly do not allow for time-dependent or location-specific *in vivo* measurements. As the uptake and distribution of this extremely toxic metal are not understood, highly sensitive and noninvasive methods are needed for its detection in a living organism.

Early biosensors have been developed to allow for the detection of mercury in contaminated cells. For example, the mercury-binding MerR-transcription factor has been employed successfully to induce the expression of selected reporter proteins such as lux, β -galactosidase, or GFP upon exposure to the metal (Geiselhart et al. 1991; Selifonova et al. 1993; Yu et al. 1994; Lyngberg et al. 1999). The expression of such biosensors in various organisms can thus be used specifically to detect mercury in contaminated environments (Klein et al. 1997; Bontidean et al. 2004). Since the reported signal, however, is not directly correlated with the amount of mercury present, a direct quantization or localization of it within a cell or tissue is precluded. Even though this detection system is highly sensitive to small amounts of the metal, the detection delay caused by the induction of the reporter gene is not suitable to monitor the immediate, time-resolved uptake of the metal by an organism.

Recently, a foldamer-based mercury sensor was reported that is based on fluorescence-energy-transfer reporter groups (Zhao and Zhong 2006). Upon binding to mercury, the polymer sensor folds into a compact structure that brings the attached FRET chromophores into close proximity. This sensor was shown to be widely responsive over five orders of magnitude of mercury concentrations.

In alternative approaches, enzymes, metal-binding proteins, as well as antibodies have been developed to facilitate the detection of heavy metals via activity-inhibition assays. It was shown that such protein-based biosensors when interfaced with amperometers, colorimeters, or calorimeters allow for an automated and direct read-out of the signal (Svobodova et al. 2003). Also recently, monoclonal antibodies have been developed that are capable of binding a mercury-binding fluorescent stilbenyl boronic acid cofactor (Matsushita et al. 2005). The construction of these very efficient sensors resembles the structure of a natural protein with a bound cofactor. In contrast to biosensors, however, chemically synthesized sensors need to be soaked into an organism in order to enable measurements. Even though the fluorescence activity of the antibody-cofactor complex allows for the direct read-out, this sensor, also, cannot be utilized for non-invasive monitoring of heavy metal uptake.

In this study, we describe the engineering of a fluorescent mercury biosensor that changes fluorescence intensity directly and rapidly upon the binding of mercury. The design of this biosensor is based on the green fluorescent protein (GFP) that allows for a direct physical linking of the sensor and reporter functions. The structure of this protein and its spectral properties are very well characterized and thus provide an advantageous system for the development of a metal-specific biosensor (Cubitt et al. 1999). Additionally, it is not dependent on auxiliary prosthetic groups or chromophores to fluoresce and can therefore be easily expressed in many different species. In contrast to many fluorescing chemosensors, this biosensor can be targeted to specific subcellular locations to facilitate direct measurements of cellular- and organelle-specific mercury uptake (see, e.g., Llopis et al. 1998). A GFP-based mercury sensor may thus offer unprecedented characterizations of tissues or parts of a cell that are most affected by mercury exposure.

Conversion of the GFP into a mercury biosensor

The GFP protein is a symmetrically shaped, 11-stranded β -barrel with a chromophore in its center (Cubitt et al. 1995; Ormö et al. 1996; Tsien 1998). The chromophore is formed autocatalytically through backbone cyclization involving residues S65, Y66, and G67. Previous studies have shown that a number of selectively placed mutations around the chromophore result in specific fluorescence changes, thus demonstrating the protein's potential for the development of a biosensor (Wachter et al. 2000; Hanson et al. 2002).

In this study, we have focused on engineering a metal-binding site in close proximity to the GFP chromophore. Two independent studies suggest that ionic solutes have access to the protein core and provide a basis for the

design. The crystal structures of a halide-soaked variant of the GFP demonstrate that ionic solutes do, in fact, have access to the core of the protein (Wachter et al. 2000) and therefore support the design of a metal-specific binding site in the vicinity of the chromophore. Another study also shows that zinc and copper ions can diffuse into the protein and bind specifically to a reengineered version of the protein's chromophore (Barondeau et al. 2002).

In accordance with these studies, we have focused on the two core mutations, T203C and S205C. The binding of mercury to sulfhydryl groups in proteins is extremely efficient and is likely to be the main cause for its toxicity. As mercury is routinely used in crystallography to derivatize protein molecules in crystals, the engineering of cysteine residues has been explored in great detail (Price and Nagai 1995). In previous studies, threonine at 203 and serine 205 were shown to be involved in a precisely defined hydrogen bond network involving the hydroxyl group of the chromophore. We hypothesize that any changes within this network due to the binding of a mercury ion are likely to influence the electronic environment and thus the protein's spectroscopic properties. Model building suggests that sufficient space is available for the binding of a mercury atom to either of the two engineered cysteines.

Results

Individual expressions of the 203C and 205C, the enhanced GFP (eGFP) variants, resulted in monodisperse, soluble protein as judged by native-gel electrophoresis and size-exclusion chromatography. Visual inspection of the purified eGFP205C protein already suggested altered fluorescence properties as judged by a differently colored appearance of the protein solution.

Spectroscopy

Compared with the eGFP protein, the absorption spectrum of the purified eGFP205C exhibits a significantly red-shifted absorption maximum from 489 nm to ~498 nm (Supplemental Fig. S1). Comparison of the fluorescence spectra of the two variants, on the other hand, shows that a maximum is maintained at ~509 nm with no significant changes (Fig. 1). eGFP203C, on the other hand, at 75 nM concentration displays reduced fluorescence intensity upon mercury addition (200 μ M). As all mutant proteins continue to quench fluorescence at very low pH, we have focused all spectroscopic characterizations on a pH range from 7.0 to 8.0 as this range is most relevant for the creation of an *in vivo* sensor.

In order to identify any specific affinities of the mutant proteins to bind metal ions, we have measured the fluorescence of eGFP and the eGFP205C and eGFP203C variants upon incubation with FeCl_3 , $\text{Co}(\text{NO}_3)_2$, ZnCl_2 ,

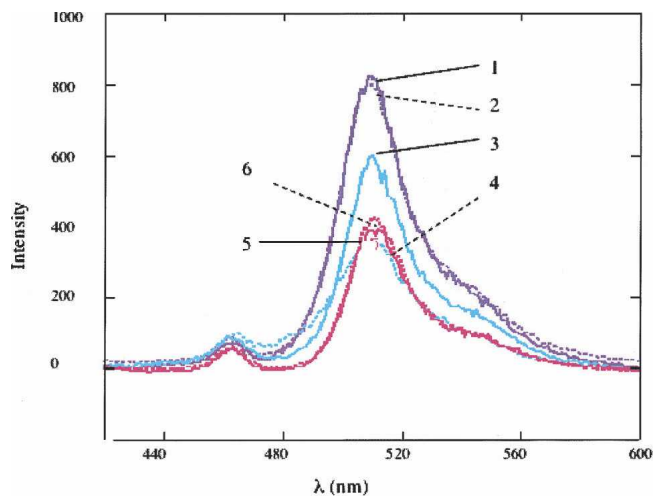


Figure 1. Comparison of the fluorescence spectra of the eGFP, 203C, and 205C proteins in response to the addition of 20 μ M HgCl_2 (in standard buffer at pH 8). The protein samples were excited at $\lambda = 400$ nm. The protein concentration was adjusted to 75 nM. In accordance with the absorbance spectra, only the 205C shows a marked decrease of fluorescence. 1 indicates eGFP; 2, eGFP + 200 μ M HgCl_2 (dashed line); 3, eGFP205C; 4, eGFP205C + 200 μ M HgCl_2 (dashed line); 5, eGFP203C; and 6, eGFP203C + 200 μ M HgCl_2 (dashed line).

HgCl_2 , CuSO_4 , FeCl_2 , NiCl_2 , and $\text{Cd}(\text{NO}_3)_2$, all at 50 μ M concentration. Significant fluorescence quenching was observed for eGFP205C only when the protein was exposed to ionic mercury by ~70% (Fig. 2). Characterization of the fluorescence properties of eGFP205C with respect to systematically varied HgCl_2 concentrations indicates a lower detection limit in the low nanomolar range (Fig. 3). Treatment of the mercury derivatized protein with metal-binding agents such as β -mercaptoethanol, glutathione, or EDTA does not regenerate the fluorescence of the protein, suggesting tight binding of the metal to the protein under these conditions.

The fluorescence results, therefore, strongly demonstrate the specificity and sensitivity of this protein for the mercury. The alternatively placed cysteine of mutant 203C also exhibits a significant response to mercury binding but with a much less stringent specificity. With the substitution of a threonine to a cysteine, a larger residue is replaced by a smaller one. This substitution may therefore create additional space that may allow other metal species to bind indiscriminately. At very high concentrations of mercury (>200 μ M), fluorescence of all tested eGFP variants vanishes completely, which is in agreement with earlier findings (Palm et al. 1997).

Mass spectrometry of *in vitro* mercuriated sensor protein

The engineered GFP sensor bears three cysteine residues including 205C that can possibly bind mercury. In order

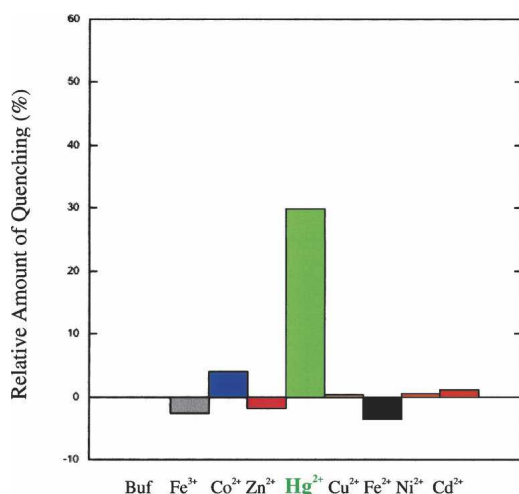


Figure 2. Fluorescence of eGFP205C with selected metals (left to right; buffer control, Fe³⁺, Co²⁺, Zn²⁺, Hg²⁺, Cu²⁺, Fe²⁺, Ni²⁺, Cd²⁺) at 1 μ M concentration each. All measurements were performed in standard buffer at pH 8. The protein concentration for all measurements was set to 75 nM. Plotted is the difference of the eGFP205C fluorescence in the absence of metal minus its fluorescence in the presence of the corresponding metal. Only mercury displays a significant drop in fluorescence.

to assess the stoichiometry of binding and to identify whether the binding of the metal in the vicinity of the chromophore induces any other covalent changes, the sensor protein was subjected to mass analysis by LC-coupled QTOF mass spectrometry. Mass analysis of the purified eGFP205C mutant reveals a mass of 26,081 Da (peak B) in the absence of mercury (without HgCl₂). The expected molecular mass for the protein, however, is 26,882.3, suggesting that six amino acids from the C-terminal end of the protein were cleaved off ($M_r = 26,086.4$ including the loss of a water molecule and two protons due to chromophore formation). A second smaller peak (peak A) is also observed, consistent with the cleavage of the N-terminal methionine.

According to the procedure described above, the isolated eGFP205C protein was also incubated with 5 μ M of mercury chloride. Under these conditions, the mercury binds to the eGFP205C mutant but not to eGFP, suggesting that only the newly engineered cysteine is the binding residue. The corresponding mass spectra are shown in Figure 4. A comparison between the apo-eGFP205C protein (without HgCl₂) and the mercury-eGFP205C spectra (with HgCl₂) clearly show a molecular-weight increase (of peaks A and B) by 199 mass units, suggesting the substitution of the thiol proton by a single mercury ion (the average mass of mercury is 200 u, resulting in Peaks C and D). A small peak (D, without HgCl₂ spectrum) is likely to correspond to another unidentified mass species that is present in a distinctly different region of the LC chromatogram and is therefore unlikely to be an eGFP

variant. Control tests have shown that this mass is frequently also observed in other bacterial non-GFP protein preparations as well (data not shown). All other peaks appear to remain constant in position and size when compared between the apo- and mercury-containing spectra, suggesting that no other covalent modifications are induced in response to the binding of mercury.

Structure

The crystal structure of the mutant protein in both crystal forms exhibits only minor differences compared with the eGFP protein. Structural changes are mostly observed in the loop structures of the protein that are directly involved in crystal contacts. All but one β -strand superimpose most accurately with an average root mean square deviation (RMSD) of 0.3 Å. In both crystal structures, the β -strand that carries the mutation between residues 202–206 has moved toward the center of the barrel by ~ 0.8 Å on average (Fig. 5). These structural changes are observed for all molecules of the two different crystal forms (NAT1 and NAT2; see Materials and Methods). Similar motions of this strand, however, can also be observed by superposing other GFP structures (Palm et al. 1997). The changes, therefore, cannot simply be attributed to the newly introduced mutation. The electron density of cysteine 205 reveals some conformational flexibility of its side chain orientation (Supplemental Fig. S2). The two

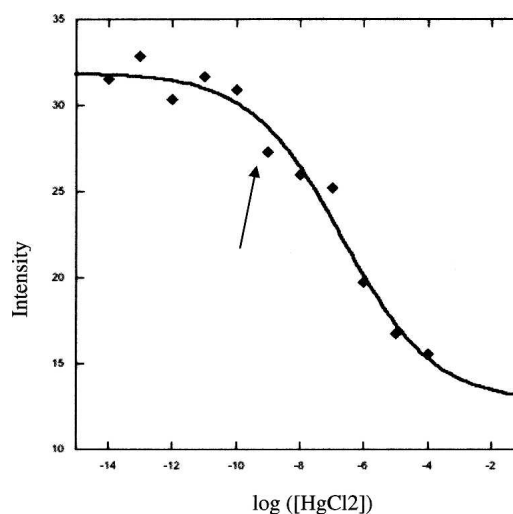


Figure 3. Fluorescence behavior of mutant 205C in response to systematically varied mercury concentrations. All measurements were performed in standard buffer at pH 8. The protein at a concentration of 0.6 nM was incubated with mercury for ~ 10 –15 min prior to data acquisition. The 205C variant exhibits a sigmoidal binding transition at low mercury concentrations, indicating a lower mercury detection limit of ~ 2 nM (corresponding data point is shown by arrow).

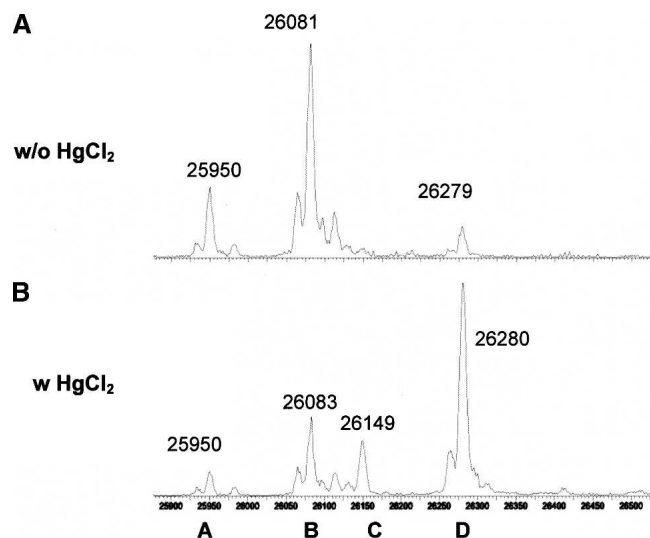


Figure 4. Mass spectra of the eGFP205C protein in the absence and presence of mercury. Peaks A and B correspond to residues 1–232 of the protein with the N-terminal methionine residue cleaved (A) or present (B). Upon the addition of ionic mercury, both mass peaks increase by 199 mass units (peaks C and D), consistent with the average mass for a single atom of mercury ($M_{\text{Hg}} = 200$ u). No other covalent modifications of the protein are apparent upon exposure to the metal.

major rotamer conformations are observed in all of the molecules of both crystal forms as judged by 2Fo-Fc maps as well as by anomalous dispersion electron density maps (data not shown).

Time-resolved characterization of mercury binding

As shown in Figure 6, the binding of mercury is a rapid process. Addition of 200 μM mercury chloride to eGFP205C in standard buffer (see Materials and Methods) resulted in rapid quenching of fluorescence, whereas the addition of the same amount to eGFP had no effect. Similarly, the addition of the same amounts of mercury to eGFP203C resulted in only minor quenching of the fluorescence, as expected (data not shown).

In vivo application of the sensor protein

To evaluate the function of the eGFP205C variant as an in vivo mercury sensor, bacterial cells overexpressing the mutant protein were exposed to 20 μM of ionic mercury in LB medium. Within seconds of exposure, the bacterial fluorescence was quenched, suggesting rapid uptake of the metal from the solution (Fig. 7). Subsequent interference contrast microscopy clearly shows that bacteria have survived the exposure to the metal. Further studies are required, however, to assess the in vivo detection limit of the sensor protein.

Discussion

The most striking finding of this study is the high specificity and affinity of the 205C mutant to bind mercury. A systematic screen for other metals clearly reveals that this mutant protein is particularly sensitive for this metal (Fig. 2). Even though eGFP bears two native cysteines at positions 48 and 70, only the newly introduced cysteine 205 converts the protein into a highly specific sensor. Incubation of eGFP with mercury results in fluorescence changes only at very high concentrations, that is, at concentrations greater than 200 μM . At lower concentrations no significant changes were observed. eGFP205C, on the other hand, exhibits significant quenching of fluorescence by relatively low concentrations of the metal, indicating a mutation-specific increase of sensitivity. As illustrated in Figure 3, systematically varied concentrations of mercury exhibit sigmoidal quenching of fluorescence; 2 nM HgCl_2 is the lowest concentration of the metal at which quenching can be observed reliably for this protein concentration. A systematic test of fluorescence of mutant eGFP203C shows that quenching can only be detected at concentrations greater than 200 μM . Direct comparison of the sensing function between the two proteins suggest a ~ 150 -fold greater sensitivity of the 205C variant at 75 nM.

Interestingly, even though the soluble protein binds Hg^{2+} very quickly and at low concentrations, it does not do so in either of the crystal forms described above. Even at relatively high mercury concentrations (>5 mM), it was

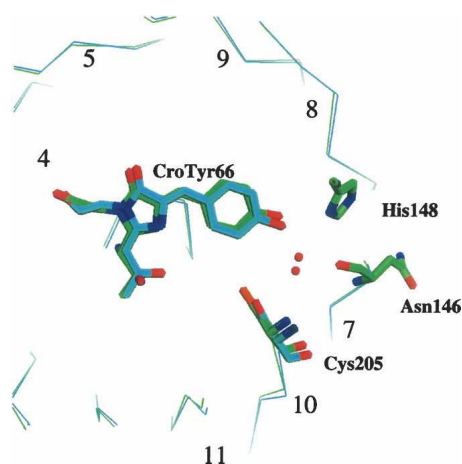


Figure 5. Superposition of structures 1EMB and molecule A of eGFP205C-NAT2. The structures are highly similar with an overall RMSD of only 0.3 Å. Only the β -strand residues 202–207 (strand 10) move toward the chromophore by ~ 0.9 Å. All other strands superpose accurately within experimental error. Similar shifts of this β -strand are also observed in other GFP structures and are most likely caused by different crystal packing conditions. In this observed structural state, mercury cannot bind to the engineered cysteine as no fluorescence change is observed upon soaking in high mercury concentrations.

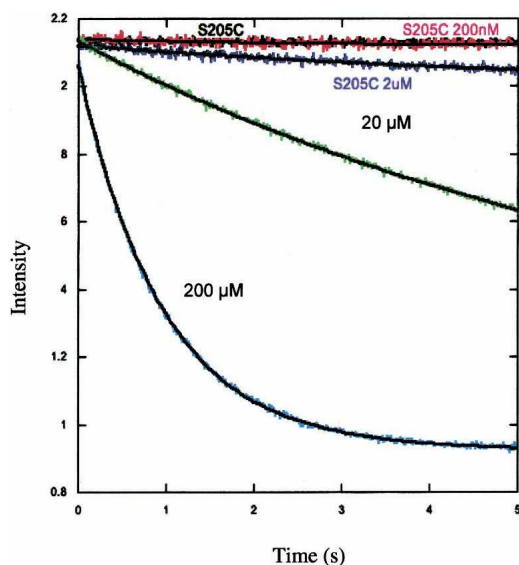


Figure 6. Time-resolved characterization of mercury binding to the 205C mutant protein via stopped-flow fluorescence spectroscopy. Protein samples at a concentration of $0.57 \mu\text{M}$ were mixed with varying concentrations of mercury chloride. Each graph represents the average of four individual measurements. We conclude that at a physiologically relevant pH (standard buffer at pH 8), the sensor activity of this mutant is sufficiently efficient to permit real-time imaging of mercury presence and dynamics. Shown are all mercury concentrations from 200 nM to $200 \mu\text{M}$. The fluorescence decays were best modeled as mono-exponential mechanisms indicating a single binding event.

not possible to derivatize the protein when the metal was soaked into the crystal as judged by a loss of fluorescence. Instead, millimolar concentrations of the metal were necessary to cause substantial discoloration of the crystals. At these high concentrations, deterioration and cracking of the crystals were also observed, which, presumably, may also be caused by the binding of mercury to the other two native cysteines.

Cysteine 205C is located in close proximity to the hydroxyl group of the chromophore tyrosine, between residues 202 and 206. The corresponding β -strand that carries this mutation forms an unusual, irregular conformation that disrupts the regular backbone hydrogen bond pattern of the β -sheet. Previous studies have suggested that the irregular conformation of the structure might allow solutes to penetrate into the core of the protein (Agmon 2005). The strong pH-sensitivity of eGFP with respect to fluorescence can be explained this way. In the crystal structures presented here, this strand and the adjacent strand bearing residues 146–149 are very tightly packed in a dimer interface. Even though the mutant protein crystallized in two different crystal forms, all molecules maintain the same characteristic dimer interface contacts (attempts to cocrystallize the protein with mercury have failed). If dynamic flexibility of the strands

is required to provide access of solutes into the core of the protein, then in the crystal this access route may now be compromised in all crystal forms. Alternatively, the conformational state of the two strands in the crystal structure may simply disfavor the binding of the metal to the cysteine for geometrical reasons. In any case, the structural flexibility of this β -strand and of the mutated residue in particular, therefore, appears to be of importance for the specific selection and binding of this metal.

Increased structural flexibility

Mutants 203C and 205C both demonstrate a substantial loss of fluorescence, which raises the question if the chromophore becomes chemically modified in response to the immediate presence of the mercury. To test this hypothesis, we have subjected the mercury-treated and the untreated 205C or 203C protein samples to mass spectroscopy and SDS-PAGE electrophoresis. None of these investigations, however, detected chain breaks or other covalent modifications. Mercury's large atomic diameter and geometry of binding are likely to require more space, as indicated by model building studies. The presence of the metal may therefore induce unfavorable interactions with its surroundings when bound to the cysteine. The solubility of the protein upon binding to the metal, however, does not change. Its increased susceptibility to proteolytic cleavage by chymotrypsin or by subtilisin, on the other hand, demonstrates an induced structural change upon binding to the metal. Whereas in the absence of mercury, the protein remains perfectly stable with respect to the protease treatment as judged by SDS-PAGE (data not shown), in the presence of the metal, however, the protein degrades rapidly (within minutes) into small peptide fragments of 8 kDa or less. Previous studies of tryptic digests of GFP also demonstrated the inherent stability of the protein against

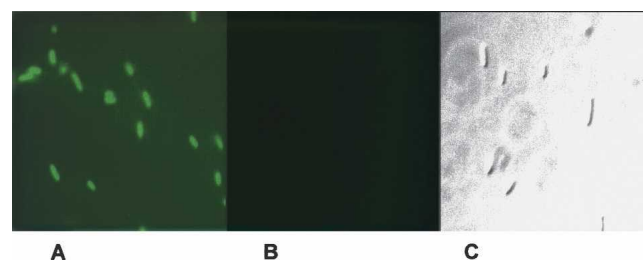


Figure 7. Fluorescence microscopy of sensor-overexpressing bacteria in response to the exposure of ionic mercury. (A) In the absence of the metal, the bacteria emit brightly. (B) Almost immediately after the addition of $20 \mu\text{M}$ of mercury, however, the fluorescence quenches sharply. (C) Subsequent observations of the bacteria in differential-interference-contrast mode suggest that bacteria have survived this treatment within the time of observation.

proteolytic cleavage (Sniegowski et al. 2005). The chymotrypsin-subtilisin digests therefore provide additional evidence that the binding of mercury may require structural changes of the protein that lead to significant destabilizations.

Stopped-flow spectrometry and microscopy

In order to assess the kinetics of mercury binding, stopped-flow binding studies were performed (Fig. 6). At all concentration tested, fluorescence quenching can be modeled best as a single exponential rate process. At a concentration of 200 μM (or above), most of the fluorescence is quenched within the first 80 ms, corresponding to a rate constant of 1.05 s^{-1} . In contrast to the previously reported biexponential fluorescence decay of halide binding (Jayaraman et al. 2000), the stopped-flow kinetics of mercury binding by eGFP205C thus suggest a single binding mechanism. The deduced binding mechanism for halide atoms suggests four equilibria involving the binding of the atom as well as the protonation of the chromophore's tyrosine hydroxyl. In contrast to this model, however, our studies show that the binding of mercury to the protein is a single kinetic event.

Given the rapid and irreversible binding of mercury to the isolated protein, this biosensor may thus be ideally suited for the time-dependent detection of ionic mercury in live organisms. The in vivo detection capabilities of this biosensor are most dramatically demonstrated in eGFP205C-overexpressing bacteria. Fluorescence-light microscopy demonstrates that with the addition of 20 μM mercury the fluorescence of the bacteria is also quenched dramatically (Fig. 7). Within the time of observation (60–120 min), exposed bacteria appeared to survive the treatment, as judged by subsequent transmission light microscopy. In order to be able to observe fluorescence changes of the sensor bacteria, these relatively high concentrations of the metal were required. We suspect that the extremely high expression of sensor protein in the bacteria artificially raises the threshold of detection. Recent test expression of the sensor protein in *Caenorhabditis elegans*, however, suggest that much lower expression levels of the protein will dramatically increase the detection limit to the nanomolar range (R.R. Chapleau and M. Sagermann, in prep.). We therefore hypothesize that a significantly reduced expression of the protein in bacteria may also improve the detection limit. Further systematic studies are required, however, to characterize this behavior and to analyze the effects of intracellular reducing agents such as glutathione on the sensor performance. The current sensitivity of the engineered biosensor, however, is suitable to detect low-level mercury contaminations that are of critical concern to human health. Due to the bio-accumulative properties of

the metal in an organism, this sensor may also be an efficient tool for monitoring the uptake and accumulation of ionic mercury in a living organism.

Conclusion

We have developed a variant of the enhanced GFP that is capable of sensing mercury ions specifically and rapidly under in vitro and in vivo conditions. Position-specific measurements of the metal within cells or tissues may be enabled via targeted expression strategies of the sensor into, for example, specific subcellular compartments or tissues. Furthermore, concentration-specific as well as time-dependent measurements of the metal may be enabled to monitor metal accumulation. To our knowledge, the use of GFP as a mercury-specific noninvasive biosensor is a first example of a real-time biosensor that may enable the direct imaging of the uptake of this metal by a living cell.

Materials and Methods

Cloning and purification

The mutations 203C and 205C were introduced using the QuikChange mutagenesis kit (Stratagene) with the following primers: 203C forward, 5'-CAACCACTACTTATCCTGTC AATCTGCCTTATCC-3'; 203C backward, 5'-GGATAAGGCA GATTGACAGGATAAGTAATGGTTG-3'; 205C forward, 5'-TACTTATCCACTCAATGTGCCTTATCCAAAGATC-3'; and 205C backward, 5'-ATCTTTGGATAAGGCACATTGATAGGA TAAGTAATG-3'. A pBAD plasmid encoding the sequence for the eGFP was used as PCR template (Cubitt et al. 1995), (Plasmid was a kind gift of Professor P.S. Daugherty of University of California, Santa Barbara). The sequence of the mutated gene was amplified and cloned into pET151 (Invitrogen) vector. Mutant eGFP proteins were overexpressed in *Escherichia coli* BL21-AI cells. For the purification of the proteins, 60 mL of cells from an overnight culture were used to inoculate 3 L of 1 mM ampicillin containing LB media. Cells were grown to mid-log phase before induction with 200 mM arabinose and 1 mM isopropyl- β -D-thiogalactopyranoside. Induction was carried out for ~3 h at 28°C in baffled, 2-L shaker flasks. After expression, cells were spun at 10,000 rpm in a Beckman JA10 rotor for ~20 min. Pelleted cells were either processed directly or stored at -70°C for later use. For the subsequent lysis, cells were thawed to $+4^\circ\text{C}$ in the presence of DNAase I and the Boeringer protease inhibitor cocktail. Approximately 20 mL of standard buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl), 1 mM EDTA, and 2 mM phenylmethanesulphonylfluoride were added to the cell suspension. Cells were then lysed using a French-press with 30 mL cell volume. The lysed cell homogenate was then spun at 17,000 rpm in a JA20 rotor for 20 min. The supernatant was used for subsequent affinity purification of the proteins via nickel affinity chromatography using standard protocols (Novagen His-tag affinity purification manual). The purity and molecular weight of the proteins were confirmed by SDS-PAGE and mass spectrometry, respectively. The purified proteins were then dialyzed overnight into standard buffer.

Crystallization

The mutant proteins were concentrated to ~10 mg/mL for overnight digestion with subtilisin in accordance with previously published protocols (Palm et al. 1997). Crystallization screens were carried out using commercially available screens (Hampton Research). Two different crystal forms were obtained using 100 mM PIPES (pH 6.5), 33% PEG 8000, and 200 mM $(\text{NH}_4)_2\text{SO}_4$ for crystal form NAT1 or using 100 mM PIPES (pH 6.5), 30% PEG 8000, and 200 mM sodium acetate for crystal form NAT2. Crystal form NAT1 belongs to spacegroup *P41212* with two molecules in the asymmetric unit, and crystal form NAT2 belongs to spacegroup *P212121* with six molecules in the asymmetric unit. As both crystal forms are non-isomorphous to any previously reported structures, molecular replacement (Collaborative Computational Project, Number 4 1994; Navaza 1994) was used to determine the structures. Refinement was carried out with the programs CNSv1.1 (Brünger et al. 1998). Details of the data collections and refinement statistics are given in the Supplemental material.

Spectroscopy

Absorption and fluorescence measurements were carried out with affinity purified protein dialyzed into standard buffer. Absorption spectroscopy was performed on a Jasco UV/Vis spectrophotometer in 100- μL volume quartz cuvettes. Fluorescence measurements were performed on a Varian fluorimeter using quartz 400- μL cuvettes. Absorption and fluorescence behavior of the proteins were monitored under systematically varied mercury concentrations (HgCl_2) ranging from 0 to 200 μM . The protein concentrations for each of the experiments are listed specifically in each of the corresponding figure legends.

Stopped-flow measurements

The time-resolved-fluorescence activity of mutant 205C upon addition of mercury was characterized on an Applied Photophysics SX18 stopped-flow fluorimeter equipped with a photomultiplier tube operated at 450 V. Twenty microliters of protein were mixed with 200 μL of standard buffer with or without HgCl_2 . The sample was excited at $\lambda = 400$ nm, and fluorescence was monitored through a 495-nm bandpass filter.

Microscopy

Fluorescence microscopy of living cells was performed on a Zeiss Axioplan fluorescing microscope. Cells overexpressing the mutant eGFP proteins were excited with a bandpass filter selected wavelength at $\lambda = 400$ nm. Two-fold concentrated mercury-containing LB media were added to the cells, and fluorescence was monitored. The vitality of the cells was monitored continuously by switching between fluorescence and transmission-light microscopy modes.

SDS-PAGE and mass spectrometry

Mutants 205C or 203C were treated with 5 μM HgCl_2 in standard buffer for ~5 min. Metal and apo-proteins were subsequently treated with subtilisin and chymotrypsin (0.1 mg/mL and 0.5 ng/mL, respectively). Thirty microliters of the digested

and nondigested controls were then mixed with 20 μL of Laemmli-loading buffer, respectively, before loading onto the gel. The masses of eGFP205C in the absence or presence of the various metal salts were determined by LC-coupled QTOF. The experimental mass accuracy was 0.02% (~2 mass units). Mutant proteins were first treated with 5 μM HgCl_2 for 5 min.

Electronic supplemental material

The supplemental material includes a figure of the absorption spectra of the protein variants, a figure of a representative electron density surrounding the mutation site, a table containing data collection and refinement statistics, experimental details for crystallography, as well as the Brookhaven Protein Data Bank identification code of the submitted protein structures.

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