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Real-time *in vivo* imaging of mercury uptake in *Caenorhabditis elegans* through the foodchain

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ABSTRACT

Mercury is a strong poison that poses significant and immediate hazards to human health. Due to its bioaccumulative properties, even small amounts of the metal are usually very poisonous or lethal when absorbed over long periods of time. Even though the possible dangers of mercury interactions with proteins are well understood, little is known about its uptake and dynamics within an organism. In particular, the concentration and distribution of the metal within a cell or a tissue are only poorly understood. In this study, we describe the application of a recently developed biosensor [Chapleau R.R., Blomberg R., Ford P.C., Sagermann M., 2008. Design of a highly specific and non-invasive biosensor suitable for real-time *in vivo* imaging of mercury(II) uptake. Protein Sci. 17(4), 614–622] that facilitates unprecedented non-invasive real-time imaging of ionic mercury uptake by an organism under *in vivo* conditions. Specifically, we here show that mercury ions can be taken up from the environment within minutes by prokaryotic as well as eukaryotic organisms. This rapid uptake can still be detected if the sensor expressing cells are shielded by layers of surrounding tissues suggesting that neither individual cell walls nor tissues provide a serious barrier for the metal. Furthermore, we show that this biosensor is suitable for the direct imaging of mercury uptake through the food chain. Our results suggest that ionic mercury remains available for extended periods of time and can rapidly contaminate surface as well as embedded tissue cells.

1. Introduction

Heavy metal poisoning remains a serious hazard in many industrialized environments. Inorganic as well as organic derivatives of the metal can readily be absorbed by inhalation or ingestion. Regardless of its chemical nature, this metal has been shown to bind to many proteins indiscriminately. Whereas the ionic form is likely to bind to protein thiols, its organometallic derivatives often bind to proteins *via* hydrophobic interactions. Due to its bioaccumulative properties, long-term exposures of even minute amounts of this metal can result in severe neurological disorders among many other diseases (Risher and De Rosa, 2007; Guzzi and La Porta, 2008).

Due to its colorless and odorless properties, the detection of mercury is challenging—particularly at nanomolar concentrations. A variety of different analytical tools and sensors have been developed that enable the detection of mercury in an organism or in the environment (Barek et al., 2001; Coronado et al., 2005; Matsushita et al., 2005; Zhao and Zhong, 2006). Whereas most of these methods are highly accurate and sensitive, they usually require invasive preparations for the quantitative or qualitative determinations of the metal. Most recently, fluorescent dyes have advanced the detection of the metal as they have pushed the detection limits into the low nanomolar ranges. Many of these compounds have also been suggested to function in cellular environments and tissues (Yoon et al., 2005; Yang et al., 2007). When soaked into a cell or organism, the dyes provide a broad picture as to how the metal is taken up by a tissue. As the dyes, however, cannot be targeted selectively to specific tissues, cells or subcellular compartments, they are unsuitable for the monitoring of real-time uptake or the cell specific distribution or dynamics within an organism. As some of these dyes may also interact unspecifically with membranes and other subcellular structures, true *in vivo* characterizations of the metal's distribution and dynamics are not possible. In particular, the hydrophobic interactions of some dyes may modify membrane permeability artificially and influence intrinsic uptake properties. For these reasons, accurate cell specific *in vivo* measurements of mercury uptake are precluded.

Biosensors are much better suited to enable *in vivo* investigations due to their less invasive properties. Protein-derived sensors, in particular, can be expressed under native conditions with little or no changes to the host cell's physiology. As the expression of protein

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sensors can be targeted to specific cells and tissues or to intracellular compartments, their expression can possibly be utilized for the location-specific uptake and accumulation of the metal.

The study presented here utilizes a recently developed biosensor that is capable of sensing ionic mercury under physiological conditions (Chapleau et al., 2008). A re-engineered version of the green fluorescent protein from *Aequoria victoria* eGFP205C was designed to detect this metal specifically and efficiently through fluorescence quenching. In contrast to other mercury biosensors, this biosensor is capable of detecting mercury in real-time to the low nanomolar concentrations. As the GFP protein has already been shown to be a versatile reporter protein for many protein-expression and protein-targeting studies (see for example Dooley et al., 2004), it is ideally suited as a template for the design of novel biosensors. As this protein can generally be expressed in many different organisms and tissues, it provides an advantageous basis for cell specific monitoring of mercury uptake. In this study, we have selectively expressed the biosensor in a prokaryote (*E. coli* K12) and in selected cells of *Caenorhabditis elegans* to monitor the uptake of ionic mercury under *in vivo* conditions.

2. Materials and methods

2.1. Cloning and transfection of C. elegans

Plasmid DNA containing eGFP205C was used as a template for PCR using 5′ and 3′ complimentary oligonucleotide primers designed with BamHI and EcoRV restriction endonuclease sites, respectively. The digested PCR product was ligated to the pPD49.78 plasmid (as available in the Fire laboratory vector kit) (plasmid was a kind gift of Prof. J. Rothman of UCSB). Young adult Bristol N2 worms were transfected in the gonad with an injection mixture of the pRF4 plasmid and the pS205C.78B plasmid containing the eGFP205C gene. Both plasmids were at an equal concentration of 100 ng/L. A nitrogen-gas injection needle was used to inject the plasmid DNA into the worms. Mutant worms were differentiated from N2 by the presence of a rolling phenotype, by which the organisms exhibited a right-handed spinning motion about the long-axis of the animal.

2.2. Prokaryotic expression of eGFP205C

E. coli bacteria containing the plasmid pET151 plasmid with the gene for eGFP205C were induced with the addition of 0.2% L-(+)-arabinose and 100 μM isopropyl-beta-D-thiogalactopyranoside when the optical density of the cell culture reached 0.5 (at 600 nm). Induction was carried out for 2 h at 28 °C.

2.3. Fluorescence spectroscopy

Determination of the bulk bacterial fluorescence as well as the fluorescence of the purified sensor protein was performed with a Varian spectrofluorimeter using 400 μL quartz cuvettes (Figures S3, S4 and S5 in Supplementary Information).

2.4. Expression of the biosensor in worms

The expression of the sensor protein in the pPD49.78 plasmid is driven by the stress promoter HSP16-2 (Tawe et al., 1998; Strayer et al., 2003). The expression was performed on plates containing worms of various growth stages. A heat-shock was carried out at 36 °C for 2 h after which the worms were allowed to recover at room temperature for another 3 h. Successful expression of the protein was monitored by fluorescence microscopy.

2.5. Microscopy

Fluorescence microscopy was performed on a Zeiss Axioplan fluorescing microscope. Bacteria and nematodes overexpressing the sensor protein were excited with a bandpass-filter selected wavelength of $\lambda = 400$ nm. The vitality of the organism was monitored continuously by alternating between fluorescence and transmission-light microscopy modes. In the time-span of the experiments, there was no death of any organisms. Images were collected using the DCView software and the parameters used for image collection (brightness, gain, exposure) were held constant for each worm.

2.6. Soaking of mercury and stressors into C. elegans

Sensor expressing worms were singled out onto LB-agar pads and immobilized with levamisol. Solutions of the various metals and stress-inducing molecules were applied to the worms by directly pipetting the solution onto the worms between the slide surface and cover slip. The worm was thus instantly immersed in the new solution by capillary action. The corresponding concentrations and identities of the compounds tested are listed in the figure legends.

2.7. Food-source contamination for nematodes

The OP50 strain of *E. coli* was used as food source for *C. elegans*. For this purpose, the bacteria were grown in mercury-free LB broth to stationary phase, harvested by centrifugation, resuspended in mercury-containing LB broth (concentrations given in figure legends) and allowed to incubate for 10 min, harvested and washed thoroughly with mercury-free LB broth to remove remaining mercury from the surrounding solution. In contrast to the direct exposure of the worms to mercury, worms were not anesthesized with levamisole in order to insure proper feeding behavior.

2.8. Integration of fluorescence intensities

Average pixel intensities of the anterior lobe of the pharynx were measured by using Adobe Photoshop 7. Each worm was treated individually. To properly observe the pharynx, an image taken 30 s post-exposure and was adjusted for brightness and contrast. Each later image for the same individual was then scaled with identical settings as the 30-s image. The average intensity of the anterior lobe area was imported into Kaleidagraph for data representation and interpretation.

2.9. Mass spectrometry

E. coli BL21-AI (pET151-eGFP205Cvector) overexpressing the sensor protein were exposed to 1 mM $HgCl_2$ for 5 min and then washed extensively with mercury-free LB broth to remove all non-bound metal. Observations by light microscopy as well as plating Hg-exposed bacteria onto LB plates devoid of the metal confirmed the survival of the cells under these conditions. Exposed and washed bacteria were subsequently lysed using CellLytic Express (Sigma), and the sensor protein was isolated rapidly using HIS-tag affinity chromatography. The purified protein was then subjected to HPLC-coupled Q-TOF mass spectrometry using an Agilent 1100 Series HPLC connected to a Micromass Q-TOF 2 mass spectrometer. Data were analyzed using the Micromass MassLynx software package.

3. Results

3.1. Specificity to sensing mercury in vitro

In order to affirm the sensor's specificity towards ionic mercury, mass spectrometric and competitive binding studies were carried out on the purified protein. To test whether the binding of other metals can possibly interfere with the binding of mercury, the sensor protein was first incubated with the metals Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+} or Cu^{2+} prior to the addition of mercury. As can be seen in Experiment 1 (Figure S5, Supplementary Information), only the addition of mercury caused a substantial decrease in fluorescence, whereas the metals alone did not exhibit any significant quenching. These results suggest, therefore, that the biosensor is highly selective for ionic mercury.

In Experiment 2, we also show that the incubation of the protein with reducing agents such as 5 mM GSH had no significant effect on its fluorescence. Interestingly, the subsequent addition of 100 μM $HgCl_2$, also, had no substantial effect on its fluorescence (see also Figure S5, Supplementary Information). The data may also suggest that GSH-mediated transmercuriations onto to the sensor's thiol can be ruled out within this short time-scale.

The effect on the fluorescence of the protein that was previously exposed to GSH, β-ME or DDT and subsequently submitted to extensive dialysis is shown in Experiment 3 (Figure S5). In either case, dialysis completely restored the ability of mercury to quench the sensor's fluorescence, indicating that the reducing agents can efficiently shield the sensor form the metal.

The mass determinations were carried out on tryptic fragments of mercury-exposed eGFP205C. As described earlier, the apo-protein is extremely stable against proteolytic degradation (Chapleau et al., 2008; Chiang et al., 2001). In contrast to the wild-type protein, however, the exposure of the sensor protein to mercury causes rapid trypsinolysis within minutes, suggesting a specific mercury-induced protease sensitivity. The digestion of the sensor protein resulted in a number fragments that could readily be identified by LC-QTOF based mass spectrometry to an accuracy

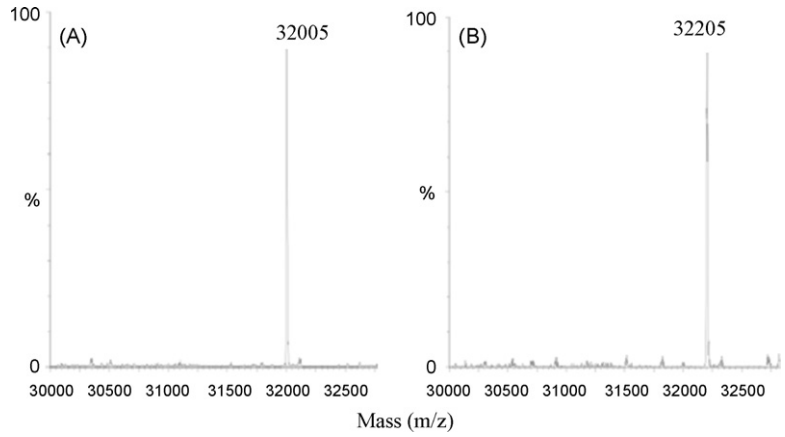

Fig. 1. Mass determination of eGFP205C from *E. coli* bacteria after HIS-tag affinity chromatography and HPLC-coupled nanoESI QTOF MS either after treatment without (a) and with 1 μM $HgCl_2$ treatment (b). The expected molecular weight of the sensor protein with its designed tag structure is 32,006 assuming complete maturation of the chromophore (which resulted in a mass decrease by 20 Da due to the loss of two protons and a water molecule).

of better than 2/10 of a mass unit. As expected, the peptide carrying cysteine 205, between residues 169 and 209, was found to have an apparent mass increase by 199 Da (total weight Mr = 4684.36 Da), suggesting the binding of a single mercury atom. The corresponding *m*/*z* values of the peptide are shown in Figure S6. Its mass, however, could only be modeled accurately, when the newly bound mercury was treated as a single positive charge on the peptide. In other words, mercury bound to 205C *via* a single covalent bond while charging the residue by one positive charge (i.e. R-Cys-Hg^+). Furthermore, none of the other two fragments that carry a native cysteine residue were found to have mercury bound. The mass spectrometric measurements, therefore, not only verify cysteine 205 as the dominant mercury binder (at these concentrations) but they also establish the valency of mercury while bound to the protein, confirming previous mass determinations of the mercuriated protein (Chapleau et al., 2008).

3.2. Performance of the biosensor in vivo

Expression of the biosensor in *E. coli* as well as in *C. elegans* resulted in brightly fluorescing organisms. In the case of the bacterial cells, the entire cell body emitted green light upon irradiation with 400 nm. Consistent with our previous findings, the addition of mercury quenched fluorescence strongly within minutes suggesting specific binding of the metal to the sensor. Whereas mercury concentrations as low as 2 nM compromised the sensor's ability to fluoresce substantially, none of the other metal species was found to quench its fluorescence at such low concentrations. In a control experiment, we also exposed wild type GFP-expressing worms to ionic mercury at 100 μM or even at 1 mM concentrations, which, however, produced no significant fluorescence decrease (S4, Supplementary Information).

As the study presented here is a first attempt to characterize its performance *in vivo*, we confirmed its binding specificity to ionic mercury by mass spectrometry (Fig. 1). Even though the bacteria were exposed to the metal for only a short period of time, almost all GFP proteins were labeled with the metal under these conditions. Attempts were also made to test the binding in *C. elegans* by mass spectrometry. Due to the low level of expression and the greatly reduced stability of the labeled protein due to proteolytic degradation, an efficient purification of the protein was not possible. The results on the mercury-exposed bacteria, however, unambiguously demonstrate the rapid uptake of the inorganic metal species by an organism and the specific binding of the metal to the sensor protein under *in vivo* conditions. It is important to note that neither the HIS-tag nor the two native cysteine residues were responsible for binding as the control protein YFP, which does not contain the 205C mutation but a HIS-tag and the cysteine residues, failed to exhibit a mass increase by 200 Da if exposed to the metal under the same *in vivo* conditions (data not shown).

3.3. Detection of mercury in C. elegans

The expression of the sensor protein in *C. elegans* was induced by a 2-h heat-shock period at 37 °C. 3 h after the induction, fluorescence was predominantly observed in the hypodermis as well as in the pharynx tissues as expected (Tawe et al., 1998; Strayer et al., 2003) (Fig. 2). Strong fluorescence was also observed in the gut granules of the central and abdominal regions of the worm. The emission of these particles, however, is routinely observed at this emission wavelength and cannot be attributed to the expression of the sensor protein. As for the bacterial cells, exposure to 2 nM $HgCl_2$ to the worms also quenched the fluorescence within minutes. As shown in Fig. 2, fluorescence decreased significantly after 75 s and ceased completely after about 4 min. In order to obtain a time-course of fluorescence quenching, a series of images were taken every 15 s and scaled with respect to another. Interestingly, the fluorescence decreased almost uniformly throughout the entire head region of the worm. No pronounced gradual disappearance of fluorescence from the hypodermis to the pharynx was observed, suggesting that the penetration of the metal through the tissues is a rather rapid process. The average amount of fluorescence was determined by integrating the pharynx region within a defined circular area on every image as shown in Fig. 2. This way several worms were treated, imaged and averaged and the results are illustrated in

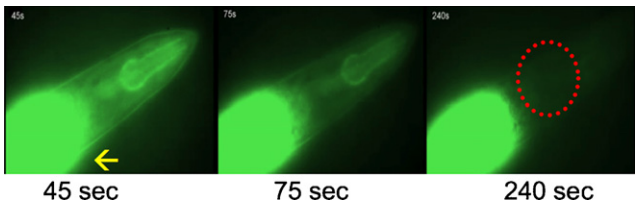

Fig. 2. Time-course of fluorescence quenching of the expressed sensor protein in *C. elegans* upon exposure to 2 nM $HgCl_2$. The pharynx and the epidermis are clearly visible after 45 s of exposure. After 75 s or later the fluorescence intensity is significantly quenched and only the gut granules (lower left of the image) fluoresce. The red dotted circle represents the radius of integration that was used to measure the overall fluorescence intensity of the pharynx within the area on every image. Image series from different and similarly sized worms were scaled and the fluorescence intensity determined (Figs. 3 and 6). (For further experimental details as well as additional movie materials, the reader is referred to Section 2 as well as the Supplementary Information.)

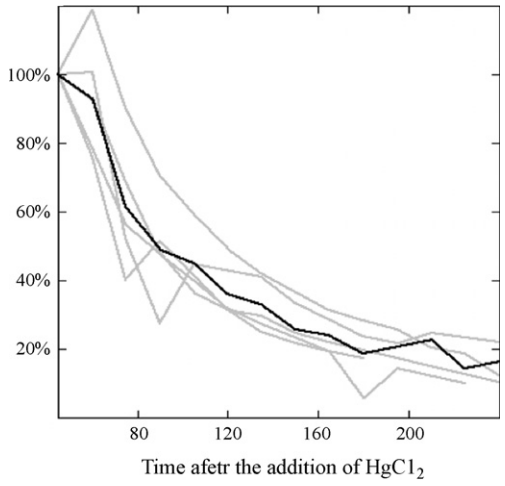

Fig. 3. Representation of the relative amount of fluorescence remaining in individual worms for the 2 nM mercury soaking experiment. The relative fluorescence intensity signal (*y*-coordinate) was obtained by integration as described in Section 2 on similarly sized and fluorescent worms. Each gray line corresponds to an individual, and the black curve is the average.

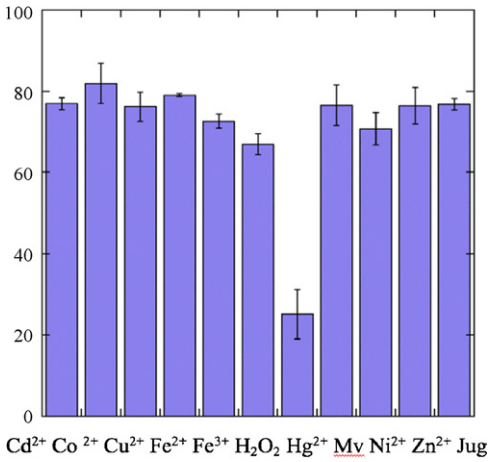

Fig. 4. Fluorescence response of *C. elegans* to selected stress-inducing compounds. Shown is the remaining fluorescence after the exposure to the compounds in percent. The concentration of mercury was 2 μM, that of juglone (Jug) was 0.2 mM, all others were at 2 mM concentration. Every compound was incubated with the worms for at least 3 min prior to fluorescence measurements. Each compound was tested on three different individual worms. Fluorescence was integrated according to the procedure described in Section 2 and averaged. The *y*-coordinate shows the fluorescent remaining after 3 min (in % compared to incubation in standard buffer). The corresponding error bars are shown in the graph. Despite a 1000-fold lower concentration, ionic mercury produced the strongest fluorescence quenching compared to all other compounds.

Fig. 3. Even though the relative sizes and expression yields are likely to be somewhat different among individual worms, they all exhibited the same dramatic loss of fluorescence upon the exposure to mercury within the same period of time. Whereas nanomolar and micromolar concentrations of the metal produced similarly strong fluorescence quenching, the addition of buffer devoid of the metal showed no significant changes.

The addition of toxic substances to a cell is known to produce rapid biochemical responses (Rico et al., 2008). Reactive oxygen species such as NO, H_2O_2 or oxygen radicals, for example, are frequently generated in response to certain stress inducers and could possibly modify thiols (Leichert et al., 2008; Lizard et al., 1998). In order to rule out binding of these compounds to the sensor, we have monitored sensor fluorescence upon the addition of selected metals and H_2O_2 *in vivo*. As can be seen in Fig. 4, none of the metals, including the most toxic metals Cd^{2+} and Cu^{2+}, produced any fluorescence changes. Thus, any small molecular compounds that might have been induced in response to the metal exposure were not interacting with the sensor.

We have also treated *C. elegans* worms with juglone or paraquat (also known as methylviologen (Mv)) at various concentrations to test the binding of endogenously produced ROS species to the sen-

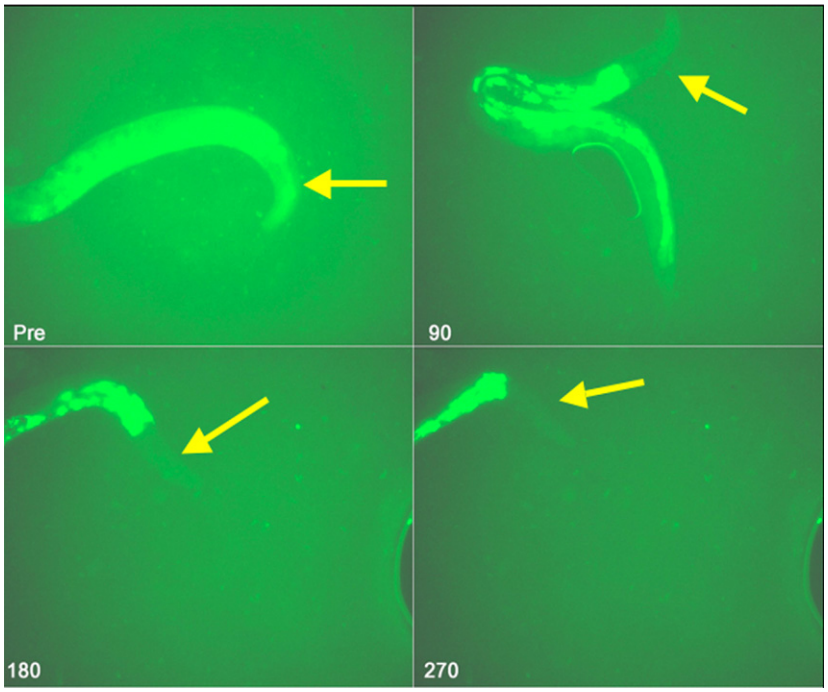

Fig. 5. Fluorescence snapshots of a feeding nematode at before (Pre) and 90 s, 180 s and 270 s respectively after the addition of contaminated bacteria. The pharynx is clearly evident before exposure to mercury, but rapidly ceases upon feeding on the bacteria contaminated with 2 μM $HgCl_2$. The time of each snapshot is indicated in the bottom left of the panel. The yellow arrow on each image points to the pharynx of the worm.

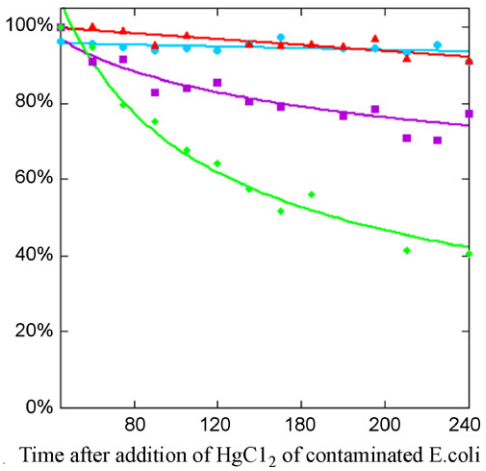

Fig. 6. Time-resolved fluorescence loss in response to mercury uptake from contaminated bacteria. The relative fluorescence (y-coordinate) at each time interval was determined by integration as described for Fig. 3. Each curve represents the average of n worms respectively. Circles = worms fed on uncontaminated bacteria ($n = 3$), or upon bacteria contaminated with 2 pM $HgCl_2$ (triangles, $n = 2$), 2 nM $HgCl_2$ (squares, $n = 4$), or 2 μM $HgCl_2$ (diamonds, $n = 6$).

sor. Both of these compounds are widely used for the induction of stress responses and the production of ROS species in *C. elegans* (Kim and Sun, 2007; Keaney et al., 2004; Kampkötter et al., 2003). Upon the addition of these compounds, however, no significant fluorescence changes of the worms were observed (Fig. 4), suggesting that the *in vivo* induction of reactive oxygen species does not affect the sensor performance. Likewise, the direct addition of H_2O_2 (0.3%), paraquat or juglone to the isolated sensor protein had also no effect on its fluorescence. Consequently, both, the *in vitro* and the *in vivo* studies therefore confirm the high specificity of the sensor protein towards binding ionic mercury exclusively.

3.4. Mercury uptake through the food-chain

In principle, the selective expression of the sensor protein in the pharynx of *C. elegans* can also be utilized to monitor the uptake of the metal through the food chain. Specifically, we monitored the fluorescence of the worms during the ingestion of bacteria that were systematically contaminated with varying concentrations of mercury prior to the feeding experiment. For this purpose, bacteria were exposed to either 0, 2 pM, 2 nM or 2 μM $HgCl_2$ containing LB medium respectively according to the above-mentioned protocol. After an extensive washing procedure, the bacteria were fed to the fluorescing worms. At various time intervals after the addition of the bacteria, fluorescence images of individual worms were taken (Fig. 5). Whereas bacteria exposed to 0- or picomolar mercury produced no significant fluorescent changes, bacteria exposed to nanomolar and micromolar concentrations of the metal produced a considerable fluorescence decrease within minutes (Fig. 6). While bacteria contaminated with a 2 nM mercury solution reduced worm fluorescence by approximately 20%, bacteria exposed to a thousand fold higher concentration exhibited quenching by about 55% after 3 min.

The observed fluorescence decrease thus appears to be dependent on the degree of contamination of the bacteria. In contrast to the mercury soaking experiment (at 2 nM $HgCl_2$), which induced a fluorescence decrease of approximately 80%, the feeding on the 2 nM exposed bacteria induced resulted in only a 20% drop of fluorescence. Even though no accurate measurements of the metal concentrations can be made with the current version of the biosensor, the data suggest that smaller concentrations of the metal have been taken up by the worms. As the sensor protein is specific to the binding of ionic mercury, the feeding experiment clearly showed that ionic mercury can be taken up rapidly through the ingestion of contaminated food.

Ingested mercury is known to undergo chemical modifications inside an organism over time (Bollen et al., 2008; Ullrich et al., 2007). To assess the potential effects of organomercurials to the sensor protein, we have incubated methyl mercury and ethyl mercury (at 5 mM) with the isolated sensor protein respectively. As can be seen in Figure S3 (Supplementary Materials), except for ionic mercury, none of these mercurials was able to quench fluorescence. This observation is therefore consistent with our previous interpretation that larger compounds are less likely to bind to the engineered thiol of the sensor due to the limited space available inside the protein.

4. Discussion

One of the most unexpected results of this study was the speed by which mercury was internalized by *E. coli* as well as by the worms. The *in vivo* studies on the bacteria and on the worms shared in common that the metal is required to penetrate through the cell membrane in order to be detected by the sensor. In bacterial cells, fluorescence was visibly quenched after only 40–60 s of exposure. In this case the metal is required to permeate the outer and inner membranes as well as through the periplasmic space. In *C. elegans*, on the other hand, the sensor expressing cells were located primarily in the hypodermis as well as in cells of the pharynx (Fig. 2). Surprisingly, however, some of the pharyngeal cells that are shielded by layers of other tissue cells were also penetrated by the metal, loosing fluorescence within minutes of exposure. In both cases, low and non-lethal concentrations of ionic mercury were applied to the organisms, which suggest that the mechanisms of metal uptake are rapid and efficient. Other metals, on the other hand, did not have an effect on the sensor's fluorescence consistent with earlier findings (Chapleau et al., 2008). Both, the *in vitro* and the *in vivo* studies suggest, therefore, that eGFP205C is highly specific to ionic mercury. Even though a co-crystal structure of the sensor and mercury is not yet available, model building suggested that several residues around the newly introduced cysteine and the space available might be important for the selective affinity. In particular, the constellation of residues H145, the chromophore hydroxyl, the hydroxyls of T203 and Y145, as well as the carboxyl group of E222 might provide a unique electronic environment that contribute to the sensor's specificity of binding. Further structural studies are required, however, to confirm the function of these residues. Both, the model building studies and the mass spectrometry of the Cys205C-containing trypsin fragment (see Supplementary Information S6) are therefore consistent and suggest the covalent binding of a singly charged Hg cation.

In principle, only two mechanisms can be envisioned that allow mercury to enter an organism so efficiently: active transport or free diffusion through the lipid membrane.

Evolution has created ion pumps and channel proteins that operate with great specificity and efficiency to allow specific ions and metals to be taken up by an organism. The uptake of trace metals such as iron or copper, for example, is facilitated through specialized protein pores and transporters to maximize efficiency (Lutsenko et al., 2007; Bressler et al., 2007). As both of these metals are toxic at high concentrations, the amount of metal uptake is required to be regulated tightly. Toxic metals such as mercury have not only been shown to compromise the function of membrane proteins directly (Bhattacharya et al., 1997; Dierks et al., 1990; Herick and Krämer, 1995; Sirois and Atchison, 1996) but have also been shown to exploit naturally occurring transport systems (Savage and Stroud, 2007; Zalups and Ahmad, 2004; Bridges and Zalups, 2005). The role

and the extent to which these transport mechanisms internalize mercury, however, are not understood.

Another possibility for the metal to enter an organism is by passive diffusion through the lipid bilayer. Studies on mercury permeability of higher eukaryote cells suggest that such mechanisms may enable the metal's uptake (Cheney et al., 2008; Braeckman et al., 1998). The most direct evidence for such mechanisms comes from experiments on liposomal membranes devoid of any proteins (Gutknecht, 1981, 1983; Nakada et al., 1978). These studies imply that mercury induces changes to membrane permeability even at low concentrations enabling relatively large biomolecules such as glucose to penetrate the membrane (Nakada et al., 1978). Interestingly, the exposure of other ions such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+} exhibited no effects on the permeability of the liposomal membranes. Another surprising result of these studies was that ionic mercury and methylmercury appeared to have similarly strong effects on membrane permeability. These early studies therefore suggest, that ionic mercury, in the presence of a specific counter ion (chloride), is a highly permeant species in biological membranes. Even though no new lipid species were observed as a result of the exposure to the metal, chemical modifications were be ruled out. It therefore seems likely that mercury permeates through the membranes through the induction of structural changes of the lipids, which in turn, may increase the permeability of other molecules as well. This mercury-induced increase of permeability would also explain the rapid uptake of the metal in the two organisms examined here.

Another most surprising observation of this study was the fact that ionic mercury in contaminated food was detected at low concentrations for relatively long periods of time. Thus far, it was commonly accepted that thiols of proteins and reducing agents such as GSH are able to quench the metal readily. In fact a recent study clearly demonstrates the relation between high-sulfur containing areas such as the ocular tissue of fish larvae and the potential to immobilize mercury (Korbas et al., 2008). Membrane proteins, for example, which are likely to be early targets of mercury exposure, have not only been shown to exhibit modified channel function upon binding mercury (Zalups, 2000; Savage and Stroud, 2007) but have also been shown to alter homeostasis of specific ions (Aduayom et al., 2005; Sirois and Atchison, 1996; Kiss and Osipenko, 1994).

Even though the relative concentrations of mercury bound to thiols other than the sensor's thiol cannot be determined with this biosensor, our *in vivo* soaking studies as well as the *C. elegans* feeding experiments with Hg-contaminated bacteria clearly point to an additional hazardous property of ionic mercury: a significant fraction of this metal appeared to remain available for at least several hours and longer, which allows it to penetrate and contaminate many cells and tissues during this time. This is most dramatically shown in the food-chain experiment. The fluorescence of the worm's pharynx decreased spontaneously upon feeding on the mercury-contaminated bacteria despite the extensive washing procedures and the several hours that passed between the contamination of the bacteria and the fluorescence measurements of the worms. As the mass spectrometry and the *in vitro* GSH-shielding experiments clearly revealed that mercury is unlikely to be transferred by reducing agents to the sensor's thiol, the results strongly suggest that sufficient amounts of ionic metal remain available to rapidly contaminate the entire head of a worm upon ingestion.

With the presented study of the eGFP205C-detection performance in live cells, we have shown that the sensor protein remains highly specific and sensitive to ionic mercury. As ionic mercury is known to efficiently interact with sulfhydryls of proteins and intracellular reducing agents to convert into other chemical species, a systematic monitoring of its consumption may be enabled with this tool. A systematic expression of the sensor protein in tissues with known (or induced) high or low GSH concentrations may provide further insights into the role and efficiencies of these compounds in removing the ionic form of this metal. Furthermore, its expression in prokaryotes with functional or disabled mercury resistance genes, for example, may advance understanding on the efficiencies of specific genes to mercury detoxification and bioremediation.

The eGFP205C biosensor may also prove useful in the evaluation of metal chelating-drugs that are commonly used for the treatment of mercury poisoning. 2,3-Dimercapto-1-propanesulfonic-acid, dimercaprol, glutathione and N-acetylcysteine, for example, are routinely used compounds to scavenge such heavy metals through intravenous injection (Muran, 2006). The efficiency and extent to which these compounds are effective, however, are not understood (Risher and Amler, 2005). With the eGFP205C biosensor it may become possible to monitor the efficiencies of these compounds in binding the metal under *in vivo* conditions.

The use of this sensor protein is also envisioned to improve understanding in the distribution, turnover and uptake of the metal within a single cell. As this it can be targeted specifically to selected tissues, cells and subcellular compartments, the monitoring of the metal's uptake in different biochemical environments and compartments has become feasible. Due to the real-time sensing properties, systematic comparisons of permeabilities of selected membranes with regards to mercury uptake may be enabled in suitable live cell, vesicle and liposomal systems.

5. Conclusion

In summary, we have presented a novel tool for the non-invasive characterizations of mercury(II) uptake in a living organism. With the development of the eGFP205C biosensor, unprecedented real-time imaging of mercury uptake and dynamics have become possible. Our studies suggest that the biological membranes of *E. coli* and *C. elegans* do not present a significant barrier to this toxic metal ion.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.05.005.

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