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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