Title: Defining the Molecular-Cellular-Field Continuum of Mercury Detoxification

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Researchers working on project:

Name	Position	Current position
Susan Miller	Professor	Professor
Rachel Nauss (Calloway)	Staff research associate	Forensic analytical chemist – Albuquerque
Ian Harwood	Graduate student (PhD)	Symphony business manager
Baoyu Hong	Postdoctoral scholar	Principal scientist – Lancaster Labs
Russell Goodman	Graduate student (MS)	Coast guard
Kathryn Bewley	Postdoctoral scholar	Same
Lawrence Lee	Summer high school intern	College student
Joel D. Blum	Professor – University of Michigan	Professor – U Mich
Laura Sherman	Postdoctoral scholar – University of Michigan	AGU/AAAS Congressional Science Fellow

Executive Summary

Hg is of special interest to DOE due to past use at the Oak Ridge Reservation (ORR). Its facile redox [$Hg^{2+/0}$] chemistry, bonding to carbon [e.g. $MeHg^+$] and unique physical properties [e.g., Hg^0 volatility] underlie a complex global Hg cycle involving biotic and abiotic chemical and physical transport and transformations in soils, sediments, waterways and the atmosphere. Facultative and anaerobic bacteria make $MeHg^+$, which is neurotoxic to wildlife and humans. Sustainable stewardship requires eliminating both $MeHg^+$ and even more toxic Hg^{2+}, which is also the substrate for methylation. The proteins encoded by the *mer* locus in aerobic and facultative mercury resistant (HgR) bacteria convert soil or waterborne Hg^{2+} or $MeHg^+$ to less toxic, gaseous Hg^0. HgR microbes live in highly Hg-contaminated sites and depress $MeHg^+$ formation >500-fold in such zones. So, enhancing the capacity of natural HgR microbes to remove Hg^{2+}/$MeHg^+$ from wetlands and waterways is a logical component of contaminated site stewardship. To apply enhancement in the field requires knowing how the HgR pathway works including the metabolic demands it makes on the cell, i.e., the entire cell is the relevant catalytic

unit. HgR loci occur in metabolically diverse bacteria and unique *mer*-host co-evolution has been found. In this project we extended our previous studies of mer enzymes in γ-proteobacteria, which are abundant in high Hg areas of the ORR to include studies of mer enzymes from HgR α-proteobacteria and HgR actinobacteria, which also increase in the high Hg regions of the ORR. Specifically, we (1) examined interactions between structural compoenents of MerA and MerB enzymes from γ-proteobacteria, (2) investigated effects of mutations on kinetic efficiency of Hg^{2+} reduction by γ-proteobacterial MerA, (3) cloned and performed initital characterization of MerA and MerB enzymes from *Streptomyces lividans*, an actinobacterium, (4) cloned and performed initial characterization of a fused MerB-MerA protein from *Ochrobactrum anthropi*, an α-proteobacterium, (5) investigate the extent of Hg isotope fractionation that occurs with purified γ-proteobacterial MerA.

Accomplishments

***(1) γ-Proteobacterial MerA/MerB* – (a) Studies of NmerA with Core within full-length MerA.** In the *mer* pathway, organomercurial lyase, MerB converts RHg^{+} to Hg^{2+}, which then must be transferred to mercuric ion reductase, MerA, for reduction to Hg^{0}. In γ-proteobacterial *mer* operons, MerA has a metallochaperone-like domain, NmerA, tethered to the N-terminus of each monomer of its homodimeric catalytic core. To simplify analysis of the role of NmerA, we previously used separately expressed NmerA and catalytic core to show that NmerA can acquire Hg^{2+} from cellular proteins and deliver it to the catalytic core. However, in vivo NmerA carries out this function while remaining tethered. Thus to fully model the turnover of RHg^{+} and Hg^{2+} species by these organisms, it is essential to evaluate the rate of intramolecular transfers between the tethered domains. Beginning with work in our previous award (DE-FG02-05ER64120), we developed a method to produce pure intact full length γ-proteobacterial MerA with the NmerA domains tethered to the catalytic core. We also generated a mutant that retains only one NmerA cysteine residue and one core cysteine residue that allowed formation of a mimic of the transient complex that occurs during Hg^{2+} handoff from NmerA to core. Using these proteins we conducted a series of studies aimed at evaluating the mobility of the tethered NmerA domains and the site of interaction of NmerA with the catalytic core during Hg^{2+} handoff. These studies were pursued in conjunction with investigators from the ORNL Hg SFA (Johs, Parks, Liang, Smith) and our manuscript describing the work was published online in August 2011: A Johs, IM Harwood, JM Parks, RE Nauss, JC Smith, L Liang, SM Miller (2011) Structural Characterization of Intramolecular Hg(II) Transfer between Flexibly Linked Domains of Mercuric Ion Reductase. *J. Mol. Biol.* 413, 639-656.

In addition to the structural studies, we used steady-state assays of the γ-proteobacterial MerA to examine the kinetic advantage of tethered NmerA by comparison of rates of Hg^{2+} transfer from both a $Hg(SG)_2$ complex and from a mutant Hg-MerB complex directly to Core and via tethered NmerA in full-length MerA. A short manuscript on this work was included in the Proceedings of the 17th International Symposium on Flavins and Flavoproteins, which was published in 2013: IM Harwood, RE Nauss, SM Miller (2013) Assessment of Kinetic Advantage Provided by Tethered NmerA in Full-length pDU1358 Mercuric Ion Reductase. In *Flavins and Flavoproteins 2011*, (SM Miller, R Hille, B Palfey, eds), pp. 625-636, self-published through Lulu.com, 2013.

(b) Studies with NmerA and MerB. In work from our previous, closely related award (DE-FG02-05ER64120) that was published during the current award (Hong, et al., 2010 Biochemistry 49, 8187-8196), we showed that separately expressed NmerA removes the Hg^{2+} product from MerB at least 100-fold more rapidly than the much smaller cellular thiol glutathione.

Since Hg^{2+} is buried in the MerB structure and inaccessible to even the small NmerA protein, this suggested a specific protein-protein interaction between NmerA and MerB initiates a structural change that facilitates the transfer. We identified several residues in the MerB structure that may be involved, and during the current award, we used our previously developed fluorescence kinetics methods to examine the role of a conserved arginine that lies over the entrance to the bound Hg^{2+} product in the structure. Comparison of the kinetics of Hg^{2+} transfers from wild type and mutant Hg-MerB complexes to NmerA and glutathione indicate that the arginine acts as a gate to prevent premature release of Hg(II) into the cytoplasm, but is "triggered" to open by the NmerA domain of MerA. A manuscript on this work is in preparation. We also generated two mutations of a second residue in MerB to explore its role, but initial kinetic studies suggest this residue plays only a minor role in facilitating the transfer.

During this current award period, we also submitted a manuscript on work from a previous DOE-funded study of the effects of conserved His and Tyr residues in NmerA in modulating its cysteine thiol pKa values, which directly impact its ability to bind and transfer Hg^{2+}. The reviewers raised some concerns that required additional experiments using alternative methods to verify the results. Dr. Hong enthusiastically stepped up to conduct these experiments within a two-month period. Her results completely supported our original conclusions and led to acceptance of the manuscript for publication (Ledwidge, et al., 2010 Biochemistry 49, 8988-8998).

In one last study of NmerA with MerB, we focused attention on which residues in NmerA are important for triggering the interaction with MerB. Although we have separate structures of NmerA and MerB, the two proteins do not form stable complexes that can be isolated and crystallized. Several mutants of MerB were made with the goal of generating crosslinked complexes of the two proteins for structural analysis, but all failed because of instability of the MerB mutants to the crosslinking conditions. Thus we chose to examine mutations of three surface residues on NmerA that lie in the vicinity of the NmerA Hg^{2+}-binding cysteine pair. Stopped-flow kinetic studies of the ability of these to remove Hg^{2+} from MerB showed essentially no effect of mutation of two of the residues, but a slight decrease in kinetic efficiency of Hg^{2+} removal from MerB by a mutant of His17 in NmerA indicating this residue is important for efficient Hg^{2+} transfers both to and from NmerA. A manuscript on this work is in preparation.

(2) γ-Proteobacterial MerA – effects of mutations within Core on kinetic efficiency. In the transfer of Hg^{2+} from MerB to MerA, the NmerA domain acquires Hg^{2+} from MerB, hands it to the C-terminal (CT) Cys pair on the MerA catalytic core, which then hands it to the inner buried Cys pair where it becomes reduced. The 12-amino acid C-terminal tail (CTT) of the protein must adopt alternative conformations to present the Cys pair to NmerA at the surface of the core and then to the inner buried pair. Thus, control of the dynamic motions of this tail is proposed to be critical for efficient catalysis. Consistent with this, we previously showed that mutation of a lysine that lies near the CTT to an alanine lowers k_{cat} and decreases catalytic efficiency (k_{cat}/K_{mHg}) at high concentrations of competing glutathione ligands much more than observed in wild type enzyme. The results suggest two possible roles for the lysine that are not distinguishable from the steady-state kinetics. To gain more insight, we conducted a pre-steady state kinetic study with results that support a role for the lysine in anchoring the CTT in the binding cleft. A manuscript on this work is in preparation.

Hypothesizing that a complex network of electrostatics may modulate the CTT dynamics, we identified two additional residues to test by mutation using the Rosetta protocol FloppyTail. NMR studies using ^{13}C-methyl labeling of Leu, Ile and Val residues were conducted on wild type and two mutants to look for differences in the dynamics of the CTT. Unfortunately no residues

exhibited good parameters for measuring changes in dynamics between wild type and mutant proteins. However, a combination of steady-state, pre-steady-state kinetics and pKa titrations suggest one or both of the additional identified residues play roles in modulating Hg^{2+} transfers. A manuscript is in preparation on the work, but will likely require additional studies for completion.

(3) Actinobacterial MerA/MerB: Relatives of *Streptomyces lividans* are also found in high Hg regions of the East Fork Poplar Creek of the ORR. With this award, we initiated studies of the properties and interactions of a new set of co-evolved MerA/MerB (SLMerA and SLMerB) proteins from a *mer* operon found in these bacteria. SLMerA in this pair lacks the tethered NmerA domain, and SLMerB lacks a cysteine present in the proteobacterial MerB that is essential for rapid transfer to NmerA. However, it has a separate pair of cysteines near its C-terminus that we hypothesize is essential for organomercurial binding and specific Hg^{2+} release to SLMerA. We received a clone of SLMerA from our collaborator Altenbucher that expressed well and could be purified in a similar manner as the γ-proteobacterial MerAs. We cloned the *slmerB* gene and found that it expressed well. However, the C-terminal Cys pair in the wild type SLMerB protein, and also in an active site mutant of SLMerB we made, was found to be very sensitive to oxidation during purification. Substantial effort was invested in exploring alternative procedures to protect the reduced cysteines from oxidation during purification. The main approach that looked promising used the reagent methylmethanethiosulfonate (MMTS) to modify all cysteine thiols as disulfides upon cell lysis and then re-reduce them immediately before use in experiments by incubation with dithiothreitol. Unfortunately, modification of all of the cysteines led to protein unfolding and aggregation with contaminants making purification more difficult rather than helping. Although some preliminary results were obtained with these enzymes, the poor stability of the SLMerB protein prevented significant progress toward characterizing this pair of enzymes.

***(4) α-Proteobacterial fused MerB-MerA*:** α-Proteobacterial species are one of the more prevalent species found in high Hg regions of the ORR. Genome sequences from several species identified what appeared to be a gene encoding a novel fusion protein of the two *mer* enzymes, organomercurial lyase (MerB) and mercuric ion reductase (MerA), with MerB fused to the N-terminus of MerA (MerB-MerA) in place of the more typical NmerA domain. In our original plan, we proposed studying the fused MerB-MerA protein from *Xanthobacter autotrophicus*, but further investigation into this genome showed this organism has two copies of the fused *merBA* gene, which differ slightly, making it difficult to clone. Subsequent to writing the proposal, sequences for fused *merBA* genes from other α-proteobacterial species appeared in the databases. We selected the fused *merBA* gene from α-proteobacterium, *Ochrobactrum anthropi* since only a single copy is present in its *mer* operon and the genomic DNA was available from ATCC. Three different constructs of the gene were cloned: the full OaMerBA fusion, the N-terminal OaMerB (residues 1 – 259), and the C-terminal OaMerA (residues 261 – 745), each having a cleavable N-terminal maltose binding protein (MBP) fusion. All three constructs express well and show expected activities with either *p*-mercuribenzoic acid or Hg^{2+} substrates as appropriate. Preliminary studies suggest the appended MerB domain in the fused protein enhances the efficiency of Hg^{2+} reduction over that of the OaMerA alone when Hg^{2+} is the only substrate. Thus the fused MerB domain, which can catalyze the lyase reaction can also function like an NmerA domain. A manuscript on these initial studies is in preparation.

(5) Isotope fractionation studies – The second major goal of the Miller lab in this award was to conduct a pilot study in collaboration with Joel Blum at the University of Michigan to measure Hg isotope fractionation by purified MerA. Previous studies by Kritee et al had shown that HgR

γ-proteobacterial strains exhibited mass dependent Hg isotope fractionation during both lysis of $MeHg^+$ to Hg^{2+} and reduction of Hg^{2+} to Hg^0 leading to the hypothesis that the fractionation was primarily due to the reduction by MerA. Here we tested this hypothesis using purified full-length MerA from the pDU1358 *mer* operon originally isolated from *Serratia marcescens*, a γ-proteobacterium. The design of the experimental protocol proved quite challenging with a need to keep samples both anaerobic and in the dark until all Hg^0 was removed from them. In addition, the samples had to be shipped to the University of Michigan for the isotope analysis, which also proved challenging. However, we were able to obtain results from assays conducted under two conditions expected to be relevant to the cellular conditions under which MerA functions. Analysis of the results shows fractionation consistent with the previous results reported by Kritee et al and an unexpected result. A manuscript on this work will be submitted in the near future.

Posters, Seminars, Publications

RC Goodman, R Soinski, SM Miller. (2012) Thermodynamics and Kinetics of a Flexible C-terminal Tail are Modulated by an Electrostatic Interaction. Poster at the Joint Chemistry & Chemical Biology and Integrated Program in Quantitative Biology Graduate Program Symposium

RC Goodman, JS Mugridge, R Soinski, SM Miller. (2013) Investigation of the Structural Features Controlling the Kinetics and Thermodynamics of the Mercuric Ion Reductase Carboxy-Terminal Tail. Poster at the Biophysics Graduate Program Symposium

RC Goodman, JS Mugridge, R Soinski, SM Miller. (2013) Investigation of the Structural Features Controlling the Kinetics and Thermodynamics of the Mercuric Ion Reductase Carboxy-Terminal Tail. Poster at the Gordon Research Conference on Enzymes, Coenzymes and Metabolic Pathways

Callaway RE, Sherman LS, Blum JD, Miller SM. (2014) Stable mercury isotope fractionation by mercuric ion reductase. Poster at 36th Steenbock Symposium on Enzyme Structure and Function.

Bacteria Facing the Hg Challenge – Evolutionarily Conserved and Divergent Features of Mercury Detoxification Enzymes. Susan M. Miller, invited talk at Trends in Enzymology, Ascona, Switzerland, 2010.

What Controls Specificity of Hg(II) Transfers from MerB to NmerA vs Other Thiols. Susan M. Miller, invited talk at Oak Ridge National Laboratory, 2010.

Novel Control of Electron Flow in a Disulfide Reductase. Susan M. Miller, invited talk at Gordon Research Conference on Enzymes, Coenzymes and Metabolic Pathways, 2010.

Structural Controls of Metal Ion Transfers between Enzymes of Mercury Detoxification. Susan M. Miller, invited talk at Zing Enzymes, Coenzymes and Metabolic Pathways Conference, 2011.

Novel Path for Proton Escape Controls Electron Flow in a Redox Enzyme. Susan M. Miller, invited talk at EMBO/Trends in Enzymology, 2014.

Johs A, Harwood IH, Parks JM, Nauss RE, Smith JC, Liang L, Miller SM. (2011) Structural Characterization of Intramolecular Hg^{2+} Transfer between Flexibly Linked Domains of Mercuric Ion Reductase. *J Mol Biol* 413, 639-656. Doi: 10.1016/j.jmb.2011.08.042.

Harwood IM, Nauss, RE, Miller SM. (2013) Assessment of kinetic advantage provided by tethered NmerA in pure full-length pDU1358 mercuric ion reductase. In *Flavins and Flavoproteins 2011*. Edited by S Miller, R Hille, B Palfey. San Francisco: SM Miller, R Hille, B Palfey, Self-Published (Lulu.com).

Hong B, Miller, SM. Mutation of Arg155 to Ala in MerB Opens Access to Active Site Bound Hg^{2+} by Small Thiols, Decreasing Specificity of Hg^{2+} Transfer to NmerA. Manuscript in preparation.

Goodman RC, Soinski, R, Miller, SM. Catalytic Effects of Removing Lysine Anchor for the Flexible C-terminal Tail of Mercuric Ion Reductase. Manuscript in preparation.

Callaway RE, Sherman LS, Blum JD, Miller SM. Stable mercury isotope fractionation by mercuric ion reductase. Manuscript in preparation.

Papers Supported by Previous DOE awards that were published during this award period:

Hong B, Nauss R, Harwood IH, Miller SM. (2010) Direct Measurement of Hg(II) Removal from Organomercurial Lyase (MerB) by Tryptophan Fluorescence: NmerA Domain of Co-evolved γ-Proteobacterial Mercuric Ion Reductase (MerA) Is More Efficient than MerA Catalytic Core or Glutathione. *Biochemistry* 49, 8187-8196.

Ledwidge R, Hong B, Dötsch V, Miller SM. (2010) NmerA of Tn*501* Mercuric Ion Reductase: Structural Modulation of the pKa Values of the Metal Binding Cysteine Thiols. *Biochemistry* 49, 8988-8998.