Title: Functions, Evolution, and Application of the Supramolecular Machines of Hg Detoxification

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Executive Summary

The bacterial mercury resistance (*mer*) operon functions in Hg biogeochemistry and bioremediation by converting reactive inorganic [Hg(II)] and organic [RHg(I)] mercurials to relatively inert monoatomic mercury vapor, Hg(0). Its genes regulate expression (MerR, MerD, MerOP), import Hg(II) (MerT, MerP, and MerC), and demethylate (MerB) and reduce (MerA) mercurials. We focus on how these components interact with each other and with the host cell to allow cells to survive and detoxify Hg compounds. Understanding how this ubiquitous detoxification system fits into the biology and ecology of its bacterial host is essential to guide interventions that support and enhance Hg remediation. At a more basic level, studies of interactions between the metal ion trafficking proteins in this pathway provide insights into general mechanisms used by proteins in pathways involved in trafficking of other metal ions in cells of all types of organisms, including pathways for essential metal ions such as Cu and Zn and other toxic metal ions such as Cd. In this project we focused on investigations of proteins from *mer* operons found in gamma-proteobacteria with specific objectives to use biophysical and biochemical approaches to detect and define (1) interactions between the structural components of the key detoxifying *mer* operon enzyme, mercuric ion reductase (MerA), (2) interactions between the components of MerA and the other *mer* operon enzyme, organomercurial lyase (MerB), and (3) to investigate the structure and interactions of integral membrane transport proteins, MerT and MerC, with MerA.

(1) Interactions of NmerA with MerA Core – MerA proteins found in γ-proteobacterial *mer* operons consist of a homodimeric catalytic core with a single heavy metal binding domain appended to the N-terminus of each monomer. Partial in vivo proteolytic removal of the N-terminal domain previously led to difficulties studying the full-length protein and confusion over the function of the N-terminal domain. Results from a previous project using separately cloned components resolved some of the confusion with demonstration that the NmerA domain binds and delivers Hg(II) to the catalytic core and enhances resistance to Hg(II) in thiol-depleted cells. Additional results implicated the uniquely conserved His17 in the NmerA domain as a critical residue needed for proper interactions with the catalytic core to obtain rapid, efficient Hg(II) transfer. This observation provided the impetus for work in this project to further define the critical structural features of this interaction that lead to efficient energetics of Hg(II) transfer between the domains. Several approaches have been taken to this end. Kinetic studies with additional H17 mutants of NmerA augmented the earlier studies and are being incorporated into a combined manuscript on that work. Additional kinetic studies with the structurally homologous periplasmic Hg-binding protein MerP gave initially surprising results that provide insight into the effect of structural constraints on the kinetics of the metal ion transfers. This work is in preparation for a separate publication. Initial efforts were made to computationally dock the proteins using a rigid-body docking approach, but were unsuccessful largely because the method did not account for mobility of portions of the protein that may be necessary for interactions with the Hg-NmerA complex. Mutations of residues in the MerA core predicted from interactions in manually docked models resulted in subtly altered kinetics for Hg(II) transfer but the changes were not specific to the reaction with HgNmerA. Thus we next pursued formation of a Hg(II)-crosslinked complex of mutants of the NmerA domain and the MerA core that retained only the single cysteine in each component that was predicted from other studies to be involved in the di-thiol “handoff” complex between the two domains. While several pieces of evidence indicated that the desired complex does form, further analysis showed the reaction was either not stoichiometric or the final complex is not stable through the purification protocols. Although crystals were obtained from solutions containing both domains and the apparent complex identified by the Western analysis, only the MerA core was present in the high resolution structure. Several different experimental strategies using different mercuric ion complexes, ratios of proteins and mixing techniques were evaluated but have not yielded sufficient quantities of pure, stable complex for structural characterization in solution or by crystallization.

Full length MerA construct – In light of the difficulties described above, we refocused our studies on defining the interactions of NmerA in the context of the physiologically relevant full-length form. To do so, we designed and expressed a full-length construct with an affinity tag followed by a specific protease cleavage site fused to the N-terminus of full length MerA. Since the in vivo proteolysis of full length MerA occurs between the NmerA and Core domains, sequential purification using the affinity tag followed by size exclusion allows purification of the affinity-tagged full length MerA after which the affinity tag is removed with the specific protease to obtain full length MerA minus the tag. This strategy has provided good yields of homogeneous full length MerA that have opened new lines of investigation with the intact protein. In a new collaboration with investigators

at Oak Ridge National Laboratory that developed during the course of this project, we have obtained initial results from small angle scattering experiments, which provide the first available structural information on conformations adopted by the appended domains in this full-length protein. Mutations of various combinations of the mercury-binding cysteines are providing additional complexes for both crystallization and solution scattering studies to examine the dynamics of the conformational changes and interactions in the docked complex. Pending results of crystallization trials, we anticipate one or two manuscripts will result from this work. In addition to the solution dynamics of NmerA relative to Core and direct structural characterization of interactions between NmerA/Core, these constructs will also allow us to pursue both structural and functional studies of interactions of full-length MerA with MerB and the membrane transport protein MerT.

(2) Hg(II) transfer from MerB to MerA. Previous steady-state type studies of an organomercurial lyase (MerB) from a γ-proteobacterial *mer* operon qualitatively suggested that Hg(II) could be transferred directly between MerB and MerA. However, the use of low levels of one or both proteins in those studies and the inclusion of a small molecule thiol in the reactions obscured quantitative comparison of the possible pathways for the transfer. Based on analysis of the MerB crystal structure we developed a spectroscopic probe to monitor the kinetics of Hg(II) removal from MerB and have studied the reaction using stopped flow spectroscopy. These studies have provided important insights into the relative efficiency of direct Hg(II) transfer between the proteins compared with transfer mediated by the cellular thiol buffer, insights into which protein domains and specific residues are involved in direct transfer of Hg(II) between the proteins, and insights into possible alternative mechanisms in other pathways. One manuscript has been submitted on this work and a second is in preparation.

3) MerT and MerC, the membrane transport proteins. In collaboration with the Stroud laboratory at UCSF, we have expressed and purified integral membrane transport proteins MerT and MerC and are continuing efforts towards structural and functional characterization, including interactions with other *mer* operon proteins.

4) Novel mechanism for control of electron flow in MerA. During the course of this project a new collaboration arose with the Babbitt laboratory at UCSF. Based on a bioinformatics study of proteins structurally related to MerA, we identified a novel structural rearrangement in a MerA mutant that also occurred in another member of the same superfamily that we predicted would have an important role in regulating electron flow in the catalytic mechanism. Mutation of a residue that we predicted would be critical for the structural rearrangement allowed us to trap MerA in an intermediate that is normally only transiently formed during the reaction. We are completing refinement of crystal structures on this new mutant and are preparing a manuscript describing this novel mechanism for controlling the catalytic activity.

5) Collaboration with ORNL Hg SFA. On another front we have developed a new collaboration with the ORNL Hg Science Focus Area team in two areas: (1) small angle scattering studies to examine the interactions, dynamic properties and overall structural

changes (upon ligand binding) of MerA and MerB in solution, and (2) computational simulation of the enzymatic mechanisms of catalysis, RHg and Hg(II) transfers and molecular dynamics for MerB and MerA. As noted above, comparison of the scattering behavior of the MerA core and our new full length MerA protein is providing the first look at the structure and dynamics of this full length protein and insights into the roles the dynamic motions of the NmerA domains play in MerAs efficient handling of Hg(II) for detoxification. For the computational studies (2) we have had extensive discussions with our collaborators on interpretation of existing experimental data for MerB which helped formulate mechanistic hypotheses that were tested computationally and published recently (Parks, et al., *J Am Chem Soc (2009)* 131: 13278-13285).

Posters, Seminars, Publications

Richard Ledwidge, Xiaohua Feng, Volker Dötsch, Susan M. Miller. Roles of Conserved Y and H Residues in NmerA in Metal Ion Transfer and Recognition. Trends in Enzymology Conference, Como, Italy (June, 2006)

Susan M. Miller, Xiaohua Feng, Rachel Nauss. Exploring Protein-protein Interactions in Metal Ion Transfers Between Enzymes Involved in Mercury Detoxification. 20th Winter Enzyme Mechanisms Conference (January, 2007)

Susan M. Miller, Xiaohua Feng, Rachel Nauss, Ian Harwood, Andrew Sandstrom, Robert Stroud. Control of Hg(II) Transfers in the Bacterial Detoxification Pathway. Gordon Conference on Enzymes, Coenzymes and Metabolic Pathways (2007)

Sunil Ojha, Aiping Dong, Emil F. Pai, Patricia C. Babbitt, Susan M. Miller. Is the Lys-Glu Salt Bridge of Disulfide Reductases Just a Salt Bridge? 16th International Symposium on Flavins and Flavoproteins (June, 2008)

Sunil Ojha, Aiping Dong, Emil F. Pai, Patricia C. Babbitt, Susan M. Miller. Is the Lys-Glu Salt Bridge of Disulfide Reductases Just a Salt Bridge? Trends in Enzymology (July, 2008)

Sunil Ojha, Aiping Dong, Emil F. Pai, Patricia Babbitt, Susan M. Miller. Mutation Affecting Salt-Bridge “Flickering” Captures Transient Catalytic Intermediate in Mercuric Ion Reductase. Gordon Research Conference on Enzymes, Coenzymes and Metabolic Pathways (July, 2009)

Baoyu Hong, Rachel Nauss, Susan M. Miller. Direct Transfer of Mercuric Ions from MerB and MerA: Co-evolved Enzymes from a Mercury Detoxification Pathway. American Chemical Society (April 2010)

Protein-protein Interactions Mediate Metal Ion Transfers in Bacterial Mercury Detoxification. Susan M. Miller, ASBMB Conference, Apr 2008

Biomolecular Mechanisms of Mercury Transfers and Transformations by Proteins of the Mer Operon. Susan M. Miller, American Geophysical Union Meeting, Dec 2008

Bacteria “Staying Alive” in Toxic Waste – Mechanisms and Metal Ion Transfers in Bacterial Mercury (*mer*) Detoxification. Susan M. Miller, University of Arizona, Nov 2009

Baoyu Hong, Rachel Nauss, & Susan M. Miller. Direct Measurement of Hg(II) Removal from Organomercurial Lyase (MerB) by Tryptophan Fluorescence: NmerA Domain of Co-evolved γ-Proteobacterial Mercuric Reductase (MerA) Is More Efficient than MerA Catalytic Core or Glutathione. *J. Mol. Biol.*, *submitted.*

Baoyu Hong, Rachel Nauss, and Susan M. Miller. Cys160 is Required for Rapid Hg(II) Removal from γ-Proteobacterial Organomercurial Lyase (MerB) by NmerA Domain of Co-evolved Mercuric Ion Reductase (MerA). In preparation (first draft complete).