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Engineering MerR for Sequestration and MerA for Reduction of Toxic Metals and Radionuclides

Final Technical Report

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Executive Summary

The objectives of this project were (1) to alter a metalloregulatory protein (MerR) so that it would bind other toxic metals or radionuclides with similar affinity so that the engineered protein itself and/or bacteria expressing it could be deployed in the environment to specifically sequester such metals and (2) to alter the mercuric reductase, MerA, to reduce radionuclides and render them less mobile. Both projects had a basic science component. In the first case, such information about MerR illuminates how proteins discriminate very similar metals/elements. In the second case, information about MerA reveals the criteria for transmission of reducing equivalents from NADPH to redox-active metals. The work involved genetic engineering of all or parts of both proteins and examination of their resultant properties both in vivo and in vitro, the latter with biochemical and biophysical tools including equilibrium and non-equilibrium dialysis, XAFS, NMR, x-ray crystallography, and titration calorimetry.

With MerR we found that, despite its very sensitive and specific in vivo response to Hg, in vitro the pure protein binds many transition metals (including Cd, Cu, Ni, Pd, and Zn) and the thiophilic metalloid, As, with high affinity. We successfully made an engineered, single chain derivative consisting only of the previously identified (by us) metal binding domain (MBD) of MerR and its in vitro metal binding properties faithfully replicated those of the whole dimeric MerR protein and showed minor difference for in vivo binding of metals other than Hg. Encouraged by the performance of the engineered MBD protein, we further engineered the MBD to be expressed on the bacterial cell surface. This construct was also successful, binding Hg even in the presence of competing thiol such as glutathione with higher affinity than either of two other such constructs using other proteins reported previously. US Patent "Metal Binding Protein, Recombinant Cells and Methods", United States, 6750042 was awarded in 2004. We also demonstrated that MerR only becomes a metal-specific regulator when it is bound to its operator DNA and with a combination of genetics and NMR identified an allosteric connection between its metal binding and DNA binding domains

For MerA as a model atypical substrate, we returned to early work showing that Au(I)/(III) could be reduced by MerA in vivo. The in vivo reduction of HauCl4 was readily demonstrated for cells that were expressing either the intact mer operon or just the MerA gene itself. Transmission electron microscopy and x-ray fluorescence of cell suspensions showed Au-containing electron dense circular objects from 50 nm to 300 nm in diameter adhering to the cell surface and distributed in the medium. In vitro work with the purified MerA catalytic core domain should that MerA does accelerate reduction of Au(i)/(III) to purple colloidal Au(0). X-ray absorption fine structure spectroscopy (XAFS) showed the development of Au(0) over time with just MerA and NADPH in a buffered medium at neutral pH. The reaction is multiphasic with early NADPH consumption being followed by a slower development of the purple color. With additional tuning this system could be exploited for the production of gold nanoparticles for industrial applications. In further work we built on our prior demonstration that to be a substrate for MerA, a metal must interact with its N-terminal chaperone domain, NmerA. We raised the question of whether NmerA alone could have a function in vivo and found that while this domain expressed alone does not confer resistance to Hg, it considerably enhances the ability of the catalytic core domain to confer resistance in vivo and to accelerate Hg(II) reduction in vivo. Since all of the ligands to metals in MerA core and NmerA are the sulfhydryl amino acid, cysteine, substantial editing of these proteins would be required to accommodate the oxophilic uranyl or chromic ions. Subsequent work on MerA has been directed at its interaction with the membrane bound MerT and with organomercurial lyase, MerB.

Lastly we determined the roles of the four cysteines in MerB and completed an NMR solution structure of it, the first ever for this unique enzyme. These data, along with a 3-D crystal structure (at 2.8 Å), reveal the catalytic triad of 2 cysteines and an aspartate that are involved in removal of the carbon side chain from organomercurials such as methylmercury. Our work establishes the

foundation for enhancing MerB's activity, the major biotic agent responsible for destruction of methylmercury in the environment.

Section 1: Metalloregulator MerR

We elucidated the structural basis of MerR's affinity and specificity for Hg(II) and applied this information to devise bacterial strains that can capture Hg(II) from solution while growing and without volatilizing it into the atmosphere like the wildtype operon does.

In the first of projects we worked with the repressor-activator MerR that controls transcription of the mercury resistance (*mer*) operon. It is unusual for its high sensitivity and specificity for Hg(II) in *in vivo* and *in vitro* transcriptional assays. The metal-recognition domain of MerR resides at the homodimer interface in a novel antiparallel arrangement of α-helix-5 that forms a coiled-coil motif. To facilitate study of this novel metal binding motif we assembled this antiparallel coiled coil into a single chain by directly fusing two copies of the 48-residue α-helix-5 of MerR. The resulting 107-residue polypeptide, called the Metal Binding Domain (MBD), and wild-type MerR were overproduced and purified, and their metal-binding properties *in vivo* and *in vitro* were determined. *In vitro* MBD bound ca. 1.0 equivalent of Hg(II) per pair of binding sites, just as MerR does, and it showed only slightly lower affinity for Hg(II) than MerR. EXAFS data showed that MBD has essentially the same Hg(II) coordination environment as MerR. *In vivo,* cells over-expressing MBD accumulated 70% to 100% more ^{203}Hg(II) than cells bearing the vector alone, without deleterious effects on cell growth. Both MerR and MBD variously bound other thiophilic metal ions including Cd(II), Zn(II), Pb(II), and As(III)) *in vitro* and *in vivo*. We conclude (a) that it is possible to simulate in a single polypeptide chain the *in vitro* and *in vivo* metal binding ability of dimeric, full-length MerR, and (b) MerR's specificity in transcriptional activation does not reside solely in the metal binding step.

The second project was based on our earlier finding that metalloregulators of the MerR family activate transcription upon metal binding by underwinding the operator-promoter DNA to permit open complex formation by pre-bound RNA polymerase. Historically, MerR's allostery has been monitored only indirectly via nuclease sensitivity or by fluorescent nucleotide probes and was very specific for Hg(II), although purified MerR binds several thiophilic metals. To observe directly MerR's ligand-induced behavior we made 2-fluorotyrosine-substituted MerR and found similar, minor changes in ^{19}F chemical shifts of tyrosines in the free protein exposed to Hg(II), Cd(II) or Zn(II). However, DNA binding elicits large chemical shift changes in MerR's tyrosines and in DNA-bound MerR Hg(II) provokes changes very distinct from those of Cd(II) or Zn(II). These chemical shift changes and other biophysical and phenotypic properties of wildtype MerR and relevant mutants reveal elements of an allosteric network that enables the coordination state of the metal binding site to direct metal-specific movements in the distant DNA binding site and the DNA-bound state also to affect the metal binding domain.

To apply the MBD to bioremediation we used the previously engineered small protein embodying in a single polypeptide the metal binding domain (MBD) ordinarily formed between two monomers of MerR. We examined the physiological and biochemical properties of MBD expressed on the cell surface or in the cytosol to better understand the environments in which specific metal-binding can occur with this small derivative. Over 20,000 surface copies of MBD were expressed per *Escherichia coli* cell with metal stoichiometries of ~1.0 Hg(II) per MBD monomer. Cells expressing MBD on their surface in rich medium bound 6.1-fold more Hg(II) than those not expressing MBD. Although in nature cells use the entire *mer* operon to detoxify Hg, it was interesting to note that cells expressing only MBD survived Hg(II) challenge and recovered more quickly than cells without MBD. Cell-surface-expressed MBD bound Hg(II) preferentially even in the presence of a 22-fold molar excess of Zn(II) and when exposed to equimolar Cd(II) in addition. MBD expressed in the cystosol also afforded improved survival from Hg(II) exposure for *E. coli* and for the completely unrelated bacterium *Deinococcus radiodurans*.

Section 2: Mercuric Reductase, MerA

The ligand binding and catalytic properties of heavy metal ions have led to the evolution of metal ion-specific pathways for control of their intracellular trafficking and/or elimination. Small, molecular weight proteins/domains containing a GMTCXXC metal binding motif in a $\beta\alpha\beta\beta\alpha\beta$ fold are common among proteins controlling the mobility of soft metal ions such as Cu^{+1}, Zn^{+2}, and Hg^{+2}, and functions of several have been established. In bacterial mercuric ion reductases (MerA), which catalyze reduction of Hg^{+2} to Hg^0 as a means of detoxification, one or two repeats of sequences with this fold are highly conserved as N-terminal domains (NmerA) of uncertain function. To simplify functional analysis of NmerA, we cloned and expressed the domain and catalytic core of Tn*501* MerA as separate proteins. We show Tn*501* NmerA to be a stable, soluble protein that binds 1 Hg^{+2}/domain and delivers it to the catalytic core at kinetically competent rates. Comparison of steady-state data for full length versus catalytic core MerA using Hg(glutathione)$_2$ or Hg(thioredoxin) as substrate demonstrates that the NmerA domain does participate in acquisition and delivery of Hg^{+2} to the catalytic core during the reduction catalyzed by full length MerA, particularly when Hg^{+2} is bound to a protein. Finally, comparison of growth curves for glutathione-depleted *E. coli* expressing either catalytic core, full length or a combination of core plus NmerA shows an increased protection of cells against Hg^{+2} in the media when NmerA is present, providing the first evidence of a functional role for this highly conserved domain.

Gold is a model alternative substrate with which to probe MerA's capacity to accept alternative electron receptors. We demonstratded in vivo reduction of HauCl4 by bacterial cells that were expressing either the intact mer operon or just the MerA gene itself. Transmission electron microscopy and x-ray fluorescence of cell suspensions showed Au-containing electron dense circular objects from 50 nm to 300 nm in diameter adhering to the cell surface and distributed in the medium. With the purified MerA catalytic core domain we observed accelerated reduction of Au(I)/(III) to purple colloidal Au(0). X-ray absorption fine structure spectroscopy (XAFS) showed the development of Au(0) over time with just MerA and NADPH in a buffered medium at neutral pH. The reaction is multiphasic with early NADPH consumption being followed by a slower development of the pink-to-purple color. With additional tuning this system could be exploited for the production of gold nanoparticles for industrial applications.

Section 3: Organomercurial Lyase, MerB

The bacterial plasmid-encoded organomercurial lyase, MerB (EC 4.99.1.2), catalyzes the protonolysis of organomercury compounds yielding Hg(II) and the corresponding protonated hydrocarbon. A small, soluble protein with no known homologs, MerB is widely distributed among eubacteria in three phylogenetically distinct subfamilies whose most prominent motif includes three conserved cysteine residues. We found that the 212-residue MerB encoded by plasmid R831b is a cytosolic enzyme, consistent with its high thiol requirement in vitro. MerB is inhibited by the non-physiological dithiol DTT, but uses the physiological thiols, glutathione and cysteine, equally well. Highly conserved Cys96 and Cys159 are essential for activity, whereas weakly conserved Cys160 is not. Proteins mutant in highly conserved Cys117 are insoluble. All MerB cysteines are DTNB-reactive in native and denatured states except Cys117 which fails to react with DTNB in the native form suggesting it is buried. Mass spectrometric analysis of trypsin-fragments of reduced proteins treated with N-ethylmaleimide or iodoacetamide revealed that all cysteines form covalent adducts and remain covalently modifiable even when exposed to 1:1 PHMB prior to treatment with NEM or IAM. Stable PHMB-adducts were also observed on all cysteines in mutant proteins, suggesting rapid exchange of PHMB among the remaining protein thiols. However, PHMB exposure of reduced wildtype MerB yielded only Hg-adducts on the Cys159/Cys160 peptide, suggesting a trapped reaction intermediate. Using HPLC to follow release of benzoic acid from PHMB we confirmed that fully reduced wildtype MerB and mutant C160S can carry out a single protonolysis without exogenous thiols. On the basis of the foregoing we refined the previously proposed S_E2 mechanism for protonolysis by MerB.

Building on this biochemical and genetic analysis we used NMR spectroscopy to determine the structure of the free enzyme. We found that MerB is characterized by a novel protein fold consisting of three non-interacting anti-parallel b-sheets surrounded by 6 a-helices. Attempts to form a MerB/organomercurial substrate complex in the presence of the inhibitor DTT resulted in a very stable MerB/Hg/DTT complex. By comparing the NMR data of free MerB and the MerB/Hg/DTT complex, we identified a set of residues that likely define a Hg/DTT-binding site. These residues cluster around two cysteines (C96 and C159) that are crucial to MerB's catalytic activity. A detailed analysis of the structure revealed the presence of an extensive shallow hydrophobic groove adjacent to this Hg/DTT-binding site. This extensive hydrophobic groove has the potential to interact with the hydrocarbon moitie of a wide variety of substrates and may explain the broad substrate specificity of MerB.

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