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**Structure/Function Analysis of Protein-Protein Interactions and Role of Dynamic Motions
in Mercuric Ion Reductase**

Final Technical Report for the Period January 2001 – January 2005

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Final Technical Report for Award DE-FG03-01ER63087 “Structure/Function Analysis of Protein-Protein Interactions and Role of Dynamic Motions in Mercuric Ion Reductase”

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This grant involved studies focused on analysis of structural features of mercuric ion reductase that are hypothesized to play critical roles in the function of this protein *in vivo*. Mercuric ion reductase is the key enzyme involved in bacterial pathways for detoxification of Hg(II) and organomercurials that result in reduction of Hg(II) to the elemental state. As these pathways are being incorporated into radiation resistant and other durable species for bioremediation purposes, additional insights from structure/function information suggesting methods for enhancement of the activity may be important. Moreover, as this reductase is one of the best characterized metal ion reductases to date, in some ways it serves as a paradigm for understanding what kinds of structural features are likely to be important in other metal “handling” enzymes. In addition, it may itself be a starting place for engineering alternative specificities once we understand the interplay of structural components more completely.

The specific objectives of this work were:

- 1) to define critical structural features of mercuric ion reductase involved in handling its cognizant metal ion substrate Hg(II),
- 2) to examine the *in vitro* effects of altering these features on the catalytic efficiency of the enzyme,

- 3) to examine the *in vivo* effects of altering several of these features by modification of the *mer* operon with the aim of enhancing resistance to elevated doses of Hg(II).

Specific objective (1) is to define critical structural features of mercuric ion reductase that are involved in handling its cognate metal ion substrate Hg(II). Prior to this award, kinetic and mutagenesis studies had indicated that the protein binds Hg(II) via a pathway with two cysteines located on an apparently flexible C-terminal tail and two cysteines buried next to the FAD cofactor that mediates electron transfer from NADPH. A major aim of our work has been to obtain crystal structure data, in collaboration with the Pai lab in Toronto, for wild type and appropriate mutants of the Tn501 mercuric reductase catalytic core in an effort to visualize protein motions involved in the passage of Hg(II) through the four-cysteine ligand exchange pathway to the site of reduction, and to identify other residues of potential importance to protein motions, ligand exchange reactions, and/or reduction of Hg(II) to examine through studies of site-directed mutants. To complete the image of the pathway for passage of Hg(II) from solution to the core, we have sought to generate an interdomain Hg(II) complex between the separately expressed N-terminal metal binding domain (NmerA) and the catalytic core of Tn501 mercuric ion reductase and obtain crystal structure data for it. All of the mutants were generated in the Miller lab and most of the complexes were titrated in the Miller lab prior to being sent to Toronto for crystallization and structure analysis.

Crystallography Results – In the early stages of the award, the Toronto group established conditions for crystallization of the wild type core-MerA protein and various cysteine-to-alanine mutants alone and in complex with their substrates, inhibitors, and/or other ligands. Scheme 1 illustrates the complexes predicted to occur along the pathway based on the original crystal structure of the enzyme from *Bacillus* RC607.

The predictions were tested by collecting crystal structure data for one single Cys->Ala mutant and all possible double C->A mutants of the four cysteines in the core pathway that had been titrated with Hg(II), Scheme 2 & Table 1. The goal of mapping the successive complexes along the Hg(II) pathway has largely been met with data for three of four desired core complexes. The inner complex has proved more difficult to obtain as a homogeneous sample. At least one of the

Table 1. Cys->Ala Mutants of Tn501 Catalytic Core

Mutant Complex	Relation to Scheme 1	Observations
AACC-Hg	Outer complex	Complex visible
CACC-Hg	Handoff from outer to middle complex	Complex visible
CACA-Hg	Middle complex	Complex visible
CCAA-Hg	Inner complex	Hg(II) not well visualized; studies in solution suggests sample not stable in long term
CCAA-Cd	Inner complex	Soaked samples gave mixed results, repeating with titrated samples
CAAC-Hg	Not predicted	Hg(II) visible in crystal
ACCA-Hg	Not predicted	Complex not stable in solution and no crystals
ACAC-Hg	Not predicted	Complex not stable in solution and no crystals
AAAC-Hg-NmerA	Interdomain complex	No success yet
ACAA-NADP ⁺	Flavin-C4a thiolate adduct predicted to occur during electron transfer from flavin to bound Hg(II)	Complex visualized

the Cd(II) complexes of the CCAA enzyme that was generated by soaking of the crystals in the presence of NADPH to reduce the active site cysteines and form the metal ion binding site gave some tantalizing results in the electron density maps, but the crystals are definitely not homogeneous suggesting that soaking may not be the optimal method to obtain this complex. In the past year, we obtained spectral evidence that we can bind Cd(II) at this inner site in pre-reduced enzyme in solution and, hence, are pursuing crystallization of the complex with the hope

of obtaining a more homogeneous sample. In addition, we are attempting to make a stable crystal with another alternative metal ion bound at the inner site. After these final attempts, a manuscript detailing the structures of the successive complexes in this pathway will be submitted. The piece de resistance in this effort would be to also have a structure of the interdomain complex between NmerA and the core showing the interactions in the initial exchange of Hg(II) onto the core. Despite many attempts, we have yet to generate this, but are still pursuing two remaining ideas to achieve this goal.

In addition to mapping the mobility and positioning of the C-terminal tail as Hg(II) moves along the ligand exchange pathway, we also determined the structure of a mutant that forms a spectral intermediate believed to be an adduct of the proximal cysteine with the C4a-position of the flavin isoalloxazine ring. The electron density map for this mutant nicely confirms this hypothesis. Kinetic evidence for the reaction of the enzyme at lower pH's suggests that electron transfer from the flavin to the Hg-bound proximal sulfur in the catalytic cycle that leads to reduction of Hg(II) to Hg(0) proceeds through this type of intermediate. Thus this structure provides insights for another key step in the overall catalytic cycle of this enzyme and its close relatives in the flavin disulfide oxidoreductase family. Another very interesting protein that has been shown to form a flavin C4a-thiol adduct is the LOV domain in photoreceptors. We are currently preparing a manuscript comparing the structures of the C-4a adducts and the surrounding environments in these two proteins.

Drs. Pai and Miller recently gave an invited, "interdigitated" talk at the 15th International Symposium on Flavins and Flavoproteins (April 17-23, 2005, Hayama, Japan) entitled "Complex Colors and Colorful Complexes – The Enigma of Mercury Reduction," in which we described the spectral properties of several of the various mutants used in the crystallography and presented pictures of the colorful crystals and resulting electron density maps. The talk was well received and also elicited a couple of collaborations we are pursuing to obtain spectra of several of the crystal forms and separately to evaluate some interesting, unexplained density in one of the structures using computational methods.

As indicated below, we have recently received positive reviews on a paper for *Biochemistry* detailing the role of the NmerA domain in which we have also presented the crystal structure of the oxidized form of the wild type Tn501 catalytic core. We are currently finishing up revisions and the final deposition of the coordinates for this structure (and the coordinates for the previous structure of the *Bacillus* RC607 enzyme) and will submit that manuscript via elink when it is accepted.

Specific objective (2) is to examine the in vitro effects of altering specific structural features on the catalytic efficiency of the enzyme. This objective was a major effort in the Miller lab and was focused on two areas: (a) examining mutations of conserved residues other than the cysteines in the C-terminal region defining the Hg(II) binding pathway to investigate their roles in ligand binding and catalysis, and (b) in vitro investigation of the function of the NmerA domain and the role of conserved residues in that domain on its function. The latter focus was initially funded in part by an award from the NSF.

(a) Conserved residues in the core – The major focus in our studies of the core has been on the effects of three mutations of completely conserved residues on the kinetic and thermodynamic properties in comparison with the wild type enzyme behavior. Two of these (Y100 and Y441', Tn501 core numbering) lie near the site of reduction of Hg(II) and one (K449) lies in the entry pathway for exchange of Hg(II) ligands. We have also conducted initial studies

of another completely conserved residue (G467) that lies at the end of the flexible C-terminal segment near the two ligand exchange cysteines.

The location of conserved lysine K449 in the pathway for initial exchange of the C-terminal cysteine thiols with the $\text{Hg}(\text{SR})_2$ substrate suggested a role for the amino group in the ligand exchange mechanism either through direct participation as an acid/base catalyst or through an electrostatic interaction, both of which would help to increase the nucleophilicity of the enzyme thiols for the exchange reaction. Direct participation as an acid/base catalyst was ruled out by the essentially identical kinetic behavior of a K449R mutant. To test whether the positive charge of the amino group provides an important electrostatic interaction we made a K449A mutant with the expectation that a decrease in the thiol nucleophilicity would cause a decrease in the initial rate of the reaction of the enzyme thiol with the entering $\text{Hg}(\text{SR})_2$ substrate, i.e., a decrease in the steady-state constant $k_{\text{cat}}/K_{\text{mHg}}$. Surprisingly, under standard conditions, which include 1 mM excess glutathione in the assay, the K449A mutant showed an increased $k_{\text{cat}}/K_{\text{mHg}}$ and a decreased k_{cat} . From the recently acquired structural data for the Tn501 core, we discovered a possible alternative role for the positive charge as an electrostatic anchor to limit the mobility of the C-terminal tail in the binding trough. As the anchor is missing in the mutant, the tail may be more accessible to solvent, which could explain the increased $k_{\text{cat}}/K_{\text{mHg}}$, and could also explain the reduced k_{cat} as the residence time near the inner cysteines might be decreased. To test this further, we examined the effect of varying the concentration of glutathione (GSH) on the $k_{\text{cat}}/K_{\text{mHg}}$ in assays where $\text{Hg}(\text{SG})_2$ is the substrate. Since “binding” of the $\text{Hg}(\text{SG})_2$ substrate involves loss of a GSH, the reverse of the initial encounter reaction is dependent on the concentration of GSH, as is the second ligand exchange reaction. Thus, we hypothesized that if the C-terminal tail had become more accessible to solvent, it may also be more susceptible to reversal of the initial encounter by GSH, i.e., $k_{\text{cat}}/K_{\text{mHg}}$ would be expected to be more sensitive to the concentration of GSH in the K449A mutant than in the wild type or K449R enzymes. Indeed K449A shows a dramatically increased sensitivity in $k_{\text{cat}}/K_{\text{mHg}}$ to the concentration of GSH, consistent with an increase in the accessibility of the tail to GSH in solution. Additional experiments were conducted in the last year to rule out other mechanisms that might explain the data and a manuscript on this work will be submitted this summer.

With the intriguing kinetic results for K449A, we wanted to explore the effects of a mutation predicted to decrease the flexibility of the C-terminal tail. We were particularly interested in effects on the last four residues of the tail (CCAG) since formation and release of the $\text{Hg}(\text{II})$ complex with the two adjacent cysteines would be expected to require significant conformational flexibility in the backbone of this region. We decided to evaluate the effect of an isoleucine mutation of the C-terminal glycine (G467I) with the expectation that this very large, hydrophobic residue would tend to keep the tail in the binding trough and decrease the rotational mobility of the tail. Preliminary results with this mutant indicate that the C-terminal cysteines more readily undergo reversible oxidation/reduction reactions with the inner cysteines than observed in the wild type enzyme, which is consistent with a predicted decrease in the general mobility of the tail and a positioning of the C-terminal cysteines low in the trough near the inner cysteines. Further analysis of this very interesting mutant is needed to fully understand the effects of this mutation, but was put on hold due to a lack of manpower.

Examination of the two tyrosine residues (Y100 and Y441' in the Tn501 catalytic core sequence) that lie near the site of reduction stemmed from previous studies of Tyr to Phe mutations of the homologous residues in the *Bacillus* RC607 MerA where both mutations led to decreased steady-state activity with mutation of the Y100 homolog causing the greater loss of

activity. Steady-state analysis of our Y100F and Y441F mutations in Tn501 MerA gave similar results – Y100F exhibits < 1% wild type activity, while Y441F retains 5-10%. Examination of our recent crystal structure data for several complexes of the Tn501 catalytic core show that the hydroxyl group of Y100 is positioned above and between the thiols of one of the inner cysteines (C42) at the site of Hg(II) reduction and the first of the cysteines on the C-terminal tail (C464'). In some of the electron density maps, the hydroxyl appears to be interacting directly with the thiol of C464'. These observations suggested that this tyrosine may be involved in hydrogen bonding or even proton transfer to one or both of these cysteines at various steps of the Hg(II) transfer and reduction in order to stabilize the development of negative charge during these processes. Thus we focused our studies primarily on the Y100F mutant.

Scheme 3. Oxidized enzyme (E_{ox}) has two redox centers – the inner disulfide and FAD. Hg(II) binds to the C42C47 thiol/thiolate pair of E_{H2} after which electrons from NADPH pass through FAD (generating a transient FADH⁻) to the bound Hg(II). The protonation states shown here for Tyr100, C464' and C465' in E_{H2} and E_{H4} are hypothetical.

To evaluate the effect of the Y100F mutation in more detail, we have determined the reduction potentials, the pK_a of C464' in the oxidized enzyme, and the rates of transfer of Hg(II) from the C-terminal cysteines to the inner cysteines and of reduction of Hg(II) for the mutant in comparison with the wild type protein. As illustrated in Scheme 3, oxidized MerA has two redox centers – FAD and the disulfide of the inner cysteines (C42,C47). Addition of the first two electrons leads to reduction of the disulfide to form the binding site for Hg(II) reduction. Addition of the second two electrons leads to reduction of the FAD, which only occurs transiently in the catalytic reaction when Hg(II) is bound to C42 and C47 for reduction. With the increase in hydrophobicity of the active site expected for the Y100F mutation along with the loss of potential proton donation by the tyrosine hydroxyl, we expected that the first potential for reduction of the disulfide might be lower (more difficult) than in the wild type enzyme. To our surprise this potential is more positive in Y100F suggesting that the Y100 hydroxyl group does not interact with C42. However, examination of the pK_a of the C464' thiol in the oxidized enzymes showed that the pK_a is shifted from 6.4 in the wild type enzyme to ca. 8.0 in the Y100F mutant. Since the reduction potential is measured at pH 7.0, C464' is nearly completely protonated in the Y100F mutant but more than 70% deprotonated in the wild type enzyme. This is consistent with the more positive reduction potential for the mutant since the C464' thiol also lies in the vicinity of C42. Although not conclusive, the perturbation of the C464' pK_a by the mutation provides some evidence for interactions between the Y100 hydroxyl group and the C464' thiolate during the normal reaction. However stronger evidence that the Y100 hydroxyl helps to stabilize the C42 thiolate comes from two further observations. The first is a shift of the second potential for reduction of the FAD to a more negative value. Since this reduction is

expected to require protonation of the C47 thiolate, the more difficult reduction would be consistent with the loss of the Y100 hydroxyl group to mediate protonation of the C42 thiolate concomitant with transfer of its proton to C47. The second observation is the dramatic decrease in the rate of reduction of Hg(II) bound to the inner cysteines in the Y100F mutant compared with the wild type enzyme. A manuscript on these studies will be submitted this summer.

(b) Function of NmerA and role of conserved residues – A major aim of a previous award from NSF was to examine the function of the N-terminal domain of MerA (NmerA) and to obtain the NMR structure using the separately expressed protein. The in vitro studies established that NmerA can facilitate the efficient acquisition of Hg(II) from other proteins and delivery to the core. These results, together with the in vivo studies described below and the crystal structure of the catalytic core (which were supported by this DOE award) have been incorporated into a manuscript that has received positive reviews with requests for revision that will soon be completed. The NMR structures of both the reduced and Hg(II) bound forms of NmerA have been completed and will be presented in a manuscript along with additional studies that we completed in the last no-cost extension year of this award. In the ca. 69-amino acid sequence of NmerA, only about 10 residues are completely conserved among NmerA sequences. Most of these lie in the vicinity of the metal binding cysteines (C11 and C14). We chose to examine the effects of two of these – Y62, which is also present in a few homologs of NmerA that bind other metals such as Cu(I); and H17, which is uniquely conserved in NmerA sequences. In the structure of NmerA and other homologs, the hydroxyl group of Y62 lies near the C14 sulfur suggesting it may stabilize the thiolate form of the cysteine. H17 also lies near the metal binding loop, but based on the structure would not be expected to interact directly with the cysteines. To test the role of these residues, we made the Y62F and H17N mutants and examined the effects of the mutations on the pKa values of C11 and C14 and the effects on the kinetics of the interaction of this domain with the core. As expected from the structure, the Y62F mutation raises the pKa's of the cysteines indicating that the Tyr hydroxyl group does provide stabilization of the thiolate form in the wild type protein. In steady-state kinetics studies, the Hg(II) complex of the Y62F mutant is a poorer substrate for the core suggesting that the higher pKa may lower the rate of Hg(II) transfer to the core since the more basic thiolate is a poorer leaving group. Consistent with the steady-state, presteady-state studies of the transfer both to the core from HgNmerA and in the reverse direction indicate that only the transfer to the core is slowed while the transfer back to NmerA is similar to wild type. This latter result indicates that the change in pKa also increases the affinity of the mutant for Hg(II), which is detrimental to the efficiency of transfer needed for good catalysis in this system.

In contrast to the Y62F mutation, but also consistent with the structure, the H17N mutation has very little effect on the pKa values for the cysteine thiols of NmerA. However, the Hg(II) complex of this mutant is also a poor substrate for the core indicating that H17 has some important role in the ligand exchange process. In this case, the presteady state studies demonstrate that the second order rate of interaction of NmerA and core is decreased in both directions during Hg(II) transfer between them indicating two things. On the one hand, the mutation appears to have little effect on the affinity of NmerA for Hg(II) relative to the core since the overall equilibrium for the transfer is similar to that with wild type NmerA. However, the lower rates in both directions suggests this residue may be important for recognition or correctly orienting the two proteins for the most efficient transfer. This result forms the basis for a new line of inquiry that we have proposed for future funding of this work. The results of these

latter studies, together with the NMR structure are being combined into a publication that should be submitted in the next few weeks to Biochemistry.

Specific objective (3) is to conduct in vivo experiments primarily aimed at determining the role of the N-terminal metal binding domain (NmerA) in mercury resistance. The major hypothesis to be tested is whether NmerA is of increased importance for resistance under conditions of reduced concentrations of intracellular low MW thiols, i.e., the tripeptide glutathione in *E. coli*. To test this, the Summers lab has pursued studies to compare the Hg resistance phenotype and Hg volatilization ability of full length Tn501 MerA (Full) versus the catalytic core construct (Core) or a combination of Core plus separately expressed NmerA (from the Miller lab) in a parent *E. coli* strain and its two related glutathione deficient strains - *gshA* that makes no low MW thiol, and *gshB* that makes only the precursor dipeptide γ -glutamylcysteine.

Studies with the initial pET plasmids encoding either Full or Core MerA provided by the Miller lab showed both proteins conferred Hg resistance presumably because of the high levels of leaky expression even under noninduced conditions. However, to compare the effects in the glutathione-depleted cell lines, a different construct was needed since those cells do not contain the T7RNA polymerase gene required for use with the pET vectors. Therefore, new plasmids controlled by the TetR repressor were constructed to encode Full, Core, or Core+NmerA. The effects of these constructs on the resistance of cells to Hg(II), their ability to volatilize Hg(0), and their ability to survive and grow in the presence of Hg(II) in comparison with cells expressing the wild type *mer* operon was examined in cells with normal and altered glutathione synthesis. Although none of the constructs provides full resistance, i.e., the ability of a single cell to grow on a Hg(II) containing plate, all three constructs do lead to expression of functional proteins in the cells as evidenced by Hg(0) volatilization, and all three constructs protect cells sufficiently that they can grow in liquid media containing levels of Hg(II) that are inhibitory to growth of cells lacking any MerA protein. The most important result however is the observation that the presence of either appended NmerA in the Full length construct or the separately expressed NmerA protein along with the Core provide substantially greater protection against Hg(II) than Core alone. Together with the in vitro studies indicating NmerA can facilitate abstraction of Hg(II) from other cellular proteins, these results suggest the major role of this domain may be to protect against the detrimental effects of Hg(II) distribution that may occur when the intracellular small MW thiol pool is diminished under oxidative stresses. The evolutionary retention of this domain in MerA sequences suggests these conditions may occur quite frequently in cells dealing with Hg(II) in their environment. The results of this work are incorporated into the manuscript with the in vitro results and the core structure described above.

In addition to completing the in vivo studies of the role of NmerA in the last year, the Summers lab has also initiated a phylogenetic study of the NmerA domain and MerP proteins from the MerA operons. Comparisons of these domains/proteins with other homologs suggests that both NmerA and MerP evolved from the copper binding CopZ protein involved in copper homeostasis in some bacterial species, but by independent paths. Likewise, the data suggest that the Gram + and Gram - NmerA sequences also evolved independent from one another but appear to have co-evolved with their respective MerA core sequences. These observations suggest that there may be additional residues in NmerA that are complementary to their respective core proteins and may be important for the efficient transfer of Hg(II) between the proteins. These results provide additional background to our proposed new line of investigation

to evaluate the extent and nature of interactions between the Mer proteins that may be involved in regulating the flow of Hg(II) inside the cell during the detoxification process.

Publications (completed and in preparation)

1. R. Ledwidge, B. Patel, A. Dong, D. Fiedler, M. Falkowski, J. Zelikova, A.O. Summers, E.F. Pai, and S.M. Miller*. NmerA, the Metal Binding Domain of Mercuric Ion Reductase Removes Hg(II) from Proteins, Delivers It to the the Catalytic Core and Protects Cells Under Glutathione-Depleted Conditions. Revised to *Biochemistry*.
2. R. Ledwidge, R. Soinski, and S.M. Miller*. Direct monitoring of metal ion transfer between two trafficking proteins. Submitted to *J. Am. Chem. Soc.*
3. R. Ledwidge, V. Dötsch, and S.M. Miller*. Structural, Thermodynamic and Kinetic Characterization of Wild Type and Two Mutants of NmerA – the Metal Binding Domain of Mercuric Ion Reductase. Near submission to *Biochemistry*.
4. S.M. Miller*, R. Soinski, R. Ledwidge, A. Dong, and E.F. Pai. Catalytic Effects of Removing a Conserved Lysine Anchor for the Flexible C-terminal Tail of Mercuric Ion Reductase. In preparation for *Biochemistry*.
5. A. Dong, S.M. Miller*, E.F. Pai*. Structure and Spectral Properties of the C4a Thiol-Flavin Adduct in Mercuric Ion Reductase – Comparison with the LOV Domain C4a Adduct. In preparation.
6. A. Dong, M. Malone, R. Soinski, M.J. Falkowski, S.M. Miller*, E.F. Pai*. The Hg(II) Binding Path in Mercuric Ion Reductase – A Structural Analysis. In preparation.
7. M. Malone and S.M. Miller*. Mutation of Active Site Tyrosine (Y100F) of Tn501 Mercuric Ion Reductase Alters Rates of Hg(II) Transfer and Reduction. In preparation.
8. C. Vetriani, Y.S. Chew, S.M. Miller, J. Yagi, J. Coombs, R.A. Lutz and T. Barkay. Mercury Adaptation Among Bacteria From a Deep-Sea Hydrothermal Vent. *Appl. Env. Microbiol.* **71**, 220-226 (2005).