DOE ANNUAL REPORT 30 October 2000

PROJECT TITLE: Microbial Oxidation and Demethylation Processes in the Environmental Mercury Cycle

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PERIOD COVERED: 6 October 1999 - 29 October 2000
(final year no-cost extension granted for 1/1/00-12/3100)

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DOE AWARD NUMBER: ER 62358

UNEXPENDED FUNDS ANTICIPATED AS OF 12/31/00:

$0

RESEARCH FOCUS: Main focus for the last 2.5 years (including the present 12-month no-cost extension) has been to complete biochemical and structural characterization of the organomercurial lyase (MerB). This was the 2nd major aim of the original proposal.

Mr. Keith Pitts, graduate student, remains the mainstay of this work. Dr. Andreas Heltzel left the project in Dec 1999 to join a biotech firm in his native Germany.

TECHNICAL PROGRESS:

A. Cellular Location of MerB and its Relationship to MerA (Keith Pitts)

The cellular location of all *mer* proteins except MerB had previously been determined. Since MerB lacks a typical signal sequence, it was assumed to be a cytosolic protein. However, we now know there are several classes of proteins lacking classical signal sequences that are nonetheless exported from the cytosol by Sec-independent mechanisms. Moreover, it was not obvious how the same set of transport proteins (MerTP and C) could take up the small metallic ion Hg(II) as well as the several bulky organomercurials to which MerB confers resistance.

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To resolve this question we osmotically shocked *E.coli* cells carrying the entire broad-spectrum *mer* operon on plasmid R831b or on small plasmids carrying cloned derivatives of MerB. Using both antibody and enzyme assays, we found that MerB partitioned with MerA, the mercuric reductase, and chromosomally-encoded LacZ (both previously known to be cytosolic), but not with MerP (known to be periplasmic) (Pitts, Zeng, & Summers, revised version submitted to Appl. Environ. Microbiol. 12 Sept 00). The structural implication of this observation is that the 4 cysteine residues of MerB are very likely in the thiol form, and do not serve as disulfide bonds in the protein.

Upon learning that MerA and MerB are located in the same cellular compartment, we used the homobifunctional crosslinking agent DSS to ask whether there is any stable association between them which might channel the toxic MerB reaction product, Hg(II), directly to the reductase. In vitro DSS crosslinked MerA to itself (as expected, it is known to be a dimer) but did not crosslink MerB to itself (as expected, it is known to be a monomer). And MerB did not crosslink to MerA under conditions we used in which even the faint crosslinks of the transcriptional activator MerR to RNA polymerase can be detected{Kulkarni, 1999 #2215}.

Thus, we have established that MerB is a cytosolic enzyme whose cysteines are not involved in disulfide bonds, and it does not form a stable quaternary complex with MerA.

B. Optimized in vitro assay of MerB (Keith Pitts)

Earlier work on the enzyme {Begley, 1986 #30; Begley, 1986 #31} used a general but relatively unwieldy gas chromatography assay and we have explored spectrophotometric assay alternatives. The first of these followed the decrease in absorbance of p-hydroxymercuribenzoate (PHMB) at 240 nm and was adequate for initial activity determinations during purification. However, the high extinction coefficient of PHMB at this wavelength and the known high Km of MerB {Begley, 1986 #30; Begley, 1986 #31} made this assay unsuitable for kinetic work.

We have now developed an assay based on the Hg-thiolate charge transfer chromophore at ca. 300 nm. We have established conditions in which the extinction coefficient for RHg-Cys (where R = p-hydroxy benzoic acid) is ca. 100-fold lower than that for Cys-Hg-Cys. The increase in absorbance at 300 nm when Hg(II) is released from PHMB by MerB is linear over the range of 20 μM to 1000 μM (ranging from 0.1 to 1.8 AUFS), quite adequate for kinetic work. This assay is also generalizable to any organomercurial with a low Hg-thiolate extinction coefficient compared to the Cys-Hg-Cys extinction coefficient; thus, we will be able to use it to assess substrate specificity for a much larger variety of organomercurials (Pitts, Zeng, & Summers, revised version submitted to Appl. Environ. Microbiol. 12 Sept 00).

C. Site-Directed Mutagenesis of MerB (Keith Pitts)

A preliminary report {Moore, 1990 #1219} had indicated that altering any of MerB's cysteines resulted in loss of the organomercurial resistance phenotype, a surprising finding since only 3 of the 4 cysteines are conserved in both Gram positive and Gram negative versions of the protein. For a thorough study of the role(s) of Cys residues in MerB, we made both Ala and Ser substitutions for each of the 4 Cys residues. All mutants express protein as well as the wildtype and the protein is predominantly in the soluble form except for C117A and C117S, both of which form inclusion bodies (even under leaky, low temperature expression conditions). Cys117 is one of the 3 conserved residues and these data suggest that its presence is important for normal folding of the protein, an unexpected result since its cytosolic location makes it unlikely that MerB's cysteines are involved in disulfide bonds.

In vitro enzyme assays completed so far indicate that C96A and C159S mutants completely lack activity. C160A and C160S retain 20% and 33% of wildtype activity, respectively. Enzymatic activity of the C117 mutant proteins could not be assessed because of their tendency to form insoluble precipitates. These data are consistent with the three most highly conserved cysteines (96,117, and 159) having important roles in catalysis. C160 appears to have a less significant, but not negligible, role. Since the substitutions we made were the same ones evaluated phenotypically by others previously {Moore, 1990 #1219}, our enzyme data indicate that loss of even 70-80% of activity results in a loss of resistance to phenylmercuric acetate. This bodes well for being able to obtain and assess structurally interesting proteins of intermediate function via the genetic screens and selections proposed originally.

D. Chemical characterization of the reactivity of thiol groups in MerB (Keith Pitts)

All of the mercury resistance proteins use cysteine residues to interact with mercury compounds. Unlike other proteins, the various *mer* proteins are not inhibited by Hg, but actually carry out enzymatic processes with it. Thus, the reactivity of thiols in MerB is of paramount importance in understanding and modifying its enzymatic mechanism. Crosslinking MerB with dibromobimane which becomes fluorescent when it reacts with adjacent thiols (within ca. 8 Å of each other) resulted in fluorescent derivatives of MerB itself and of at least one of its tryptic fragments. These observations indicate that at least two of the cysteines in the protein monomer are within 8 Å of each other. Dibromobimane is currently being applied to the cysteine mutant preteins in order to sort out which cysteines is involved in the intra-monomer dibromobimane cross-links. These data on the steric accessibility of MerB's cysteines will provide useful constraints for interpretation of NMR data (see below). Additional current work with wildtype and the cysteine mutants is assessing their reaction patterns with iodoacetamide in the presence and absence of organomercurial substrate. We expect to complete a manuscript on these chemical modifications of the wildtype and mutant proteins during early winter.

E. Determination of the solution structure of MerB by heteronuclear NMR (collaboration with Jim Omichinski in Dept. of Biochemistry).

This subproject which is supported by a special structural biology supplement to the original grant (awarded in May 1999) has resulted in the production of hundreds of milligrams of pure wildtype MerB protein substituted with 15N or with 13C or both. The protein has a very highly resolved proton spectrum, consistent with a very stable fold.

Jim and post-doc Dr. Paola di Liello have completed acquisition of 80% of the necessary NMR data for solving the solution structure of the protein. Data have also been taken on all of the cysteine mutant proteins, allowing unique assignment of cysteine cross-peaks. Remaining residue assignment is currently underway as the final spectroscopic data are being accumulated for both the wildtype and mutant proteins. Some data have also been taken with various organomercurial substrates added and there is evidence for an allosteric change in MerB upon interaction with all substrates.

We expect to be able to submit for publication a manuscript on the NMR solution structure of MerB by late spring, 20001. In order to complete this part of the project, we have applied for a supplement on our newer, related DOE grant from the NABIR program on the metalloregulator, MerR. This application is pending.