Organomercurial Lyase and Mercuric Ion Reductase: Nature's Mercury Detoxification Catalysts

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Concern over our increasingly polluted environment, with its potentially deleterious effects on organisms, including humans, has sparked considerable research into the strategies by which living systems contend with toxic compounds. One area of research that has been especially prolific in recent years deals with the mechanisms by which bacteria cope with increased heavy-metal burdens.[1,2] Heavy metals are prevalent throughout the biosphere, and while some (e.g., Co, Cu, Mo, Ni, and Zn) are required in trace amounts, elevated concentrations of most are deleterious by virtue of their avid ligation to cellular components, particularly proteins.[3] Especially toxic to higher organisms are organometallics, whose lipophilic nature gives them a strong tendency toward bioaccumulation in the food chain.

Bacteria have developed several general strategies for dealing with heavy metals.[1,2] One mechanism is to reduce the uptake of a particular ion (e.g., tellurite or chromate) by altering the normal transport system (e.g., phosphate) through which the toxic ion adventitiously enters. Other resistance determinants (e.g., for cadmium and arsenate) do not alter influx, but rather encode a specific efflux system which directly competes with uptake to lower the intracellular toxic ion concentration. A third means of resistance is sequestration, analogous to the eucaryotic metallothionein system where a comparatively small protein tightly binds the harmful metal species, keeping it away from more sensitive targets. But perhaps the most interesting of all are resistance determinants that actually detoxify the harmful species by enzymatically converting it to a less deleterious form. Of such determinants, the bacterial mercury resistance pathway for conversion of organomercurials and Hg(II) to Hg(0) has been the most studied and is the best understood.

Table I
Physical and Catalytic Properties of Organomercurial Lyase and Mercuric Ion Reductase

	organomercurial lyase[a]	mercuric reductase[b]
gene	*merB*	*merA*
subunit MW	22 000	59 000
native structure	monomer	dimer
cofactors	none	FAD, NADPH
preferred substrate	R–Hg–SR′	$Hg(SR)_2$
product(s)	RH + $Hg(SR')_2$	Hg(0)
V_{max} (25 °C)	1–100 min^{-1}	340 min^{-1}
K_m (in 1 mM thiol)	0.5 mM CH_3HgCl	5 μM $HgCl_2$
rate acceleration	10^6–10^7	$>10^3$[c]

[a] Organomercurial lyase from *E. coli* R831. [b] Tn*501*-derived mercuric reductase. [c] Compared to the reoxidation of dihydroflavin ($FADH^-$) by Hg(II)–EDTA. It is not possible to determine the rate of reoxidation of $Hg^{II}(SR)_2$ because the reduction potential of this complex is below that of free $FADH^-$.

Mercurials are both widely dispersed and pervasive (an estimated 200 million tons in the biosphere),[4] but they serve no known biological function. Rather, their extremely high affinity for thiols (K_{diss} values for Hg(II)–thiol chelates under physiological conditions range from 10^{-38} to 10^{-45} M)[5] renders them exceedingly toxic to living organisms. Their potent antimicrobial properties have been exploited throughout history for both industrial and clinical purposes. One notable example was the use, spanning the four centuries prior to the discovery of penicillin, of mercury fumigations for the chronic treatment of syphilis.[6,7] Organomercurials have been widely employed as both clinical and topical antiseptics (e.g., Mercurochrome and Merthiolate) and are still in common use today in the cleaning of soft contact lenses.

The predominant mercurials with which microbes must contend are naturally derived methylmercury, aryl mercurials (from industrial and clinical applications),

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Among the topics in enzymatic reaction mechanisms that this group has pursued are investigations into the molecular mechanisms of bacterial mercury detoxification. All the authors have displayed a penchant for the mercurial temperament.

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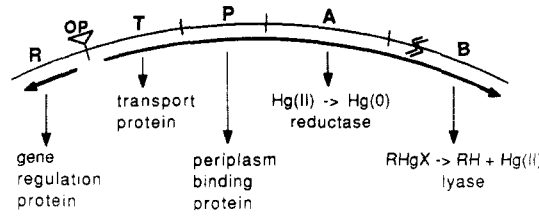


Figure 1. Typical arrangement of genes in a gram-negative *mer* operon. Letters above operon (R, T, P, and B) denote gene names (e.g., R = *merR*), while OP signifies the operator–promotor region where the MerR protein binds. Arrows designate the direction of transcription. *merB* does not occur in all *mer* operons and when present is often found very far downstream of the other structural genes.

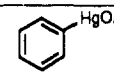
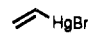
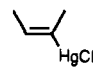
and dissolved salts of inorganic Hg(II). The most common bacterial resistance mechanism involves three distinct steps: (1) uptake, (2) cleavage of organomercurials, and (3) reduction of mercuric ion to elemental mercury. All three activities are encoded by the structural genes of the *mer* operon, which is often found associated with a plasmid and/or transposon (Tn).[8-10] To date, the gram-negative *Pseudomonas* Tn*501*[11,12] and *Shigella* Tn*21*[12,13] *mer* operons are the best characterized, with several gram-positive gene systems (e.g., *Bacillus*[14] and *Staphylococcus*[15]) having only recently been sequenced. Interestingly, all proteins expressed from the *mer* operon contain one or more sets of paired cysteines (a pair being defined as two cysteines separated by no more than five intervening residues in the primary sequence). Given the high affinity of Hg(II) for bis-thiol ligation (vide supra), it has been thought that these pairs may play key roles in the detoxification pathway.[8,11,12]

A generalized gram-negative *mer* operon is schematized in Figure 1. The MerR protein is a sensitive, Hg(II)-activated metalloregulatory switch which both positively and negatively controls structural gene transcription and autoregulates its own synthesis.[16] The structural gene products, MerP and MerT, mediate the specific uptake of mercurials.[8-10] MerP is a periplasmic mercury binding protein that putatively acts to scavenge Hg(II) and RHgX from the immediate microenvironment. MerT, which is associated with the cell membrane,[17] is thought to receive mercurials from MerP and then transfer them into the cytoplasm, where the actual detoxification takes place. The relevant catalysts are MerB, organomercurial lyase, and MerA, mercuric ion reductase (Table I).

Organomercurial lyase catalyzes the protonolytic cleavage of carbon–mercury bonds to yield the parent hydrocarbon and inorganic Hg(II) (eq 1). The resulting

$$RHgX + H^+ + X^- \rightarrow RH + HgX_2 \qquad (1)$$

Table II
Kinetic Parameters for Selected Organomercurials

substrate[a]	turnover no.[b]	substrate[a]	turnover no.[b]
CH_3HgCl	0.7		15
	5.5		
	20		12
	2.5		58
			78
	0.9		240

[a] The product in each instance is the hydrocarbon resulting from protonolysis of the carbon–mercury bond. [b] Turnover number is defined as moles of product per minute per mole of enzyme.

Hg(II) then becomes a substrate for mercuric reductase, which catalyzes the two-electron reduction of mercuric ions to elemental mercury at the expense of NADPH (eq 2). The Hg(0) so produced is considerably less toxic

$$Hg(SR)_2 + NADPH + H^+ \rightarrow Hg(0) + NADP^+ + 2RSH \qquad (2)$$

than Hg(II), and it simply volatilizes out of the cell. It is these two unique catalysts that are the focus of this Account.

Organomercurial Lyase

Organometallic species are quite rare in biological systems, and organomercurial lyase (MerB) is one of the few enzymes known to process carbon–metal bonds. The best characterized example of a carbon–metal bond in biological systems is the carbon–cobalt bond in coenzyme B_{12}.[18,19] Recently, a carbon–nickel intermediate has been implicated in the formation of acetyl-CoA from two CO_2 molecules in acetogenic bacteria. The fundamental step of that process is catalyzed by the enzyme carbon monoxide dehydrogenase, for which a biologically unique carbonyl insertion reaction (a nickel-based carbonylation) has been proposed.[20-22]

However, unlike the above carbon–metal bonds, organomercurial species are quite stable to protonolysis in aqueous solutions; therefore, the task of the MerB is not a trivial one. While the catalytic turnover of MerB is slower than most enzymatic reactions (1–100 mol of various organomercurial substrates per minute per mole of enzyme), it is still orders of magnitude faster than chemical protonolysis (10^6–10^7-fold acceleration), and there is no direct correlation between the bond strengths of various organomercurials and the enzymatic rates.[23] Even though organomercurial salts are quite resistant to chemical hydrolysis by concentrated acids[24] and bases,[25] this enzyme has evolved a mecha-

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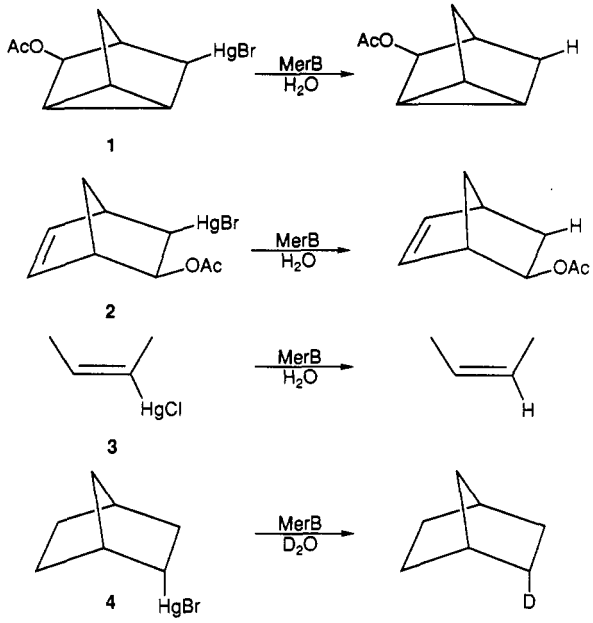

Figure 2. Radical and stereochemical mechanistic probes for MerB.

nism by which it can carry out this difficult chemical reaction in aqueous solutions at a reasonable pH (7–10) and at reasonable rates. MerB (which has 212 amino acid residues; 22 kDa) can process a broad range of substrates (alkyl and aryl organomercurials, Table II), yet it contains no metal ions or cofactors.[26] Therefore, the mechanism by which this unique enzyme cleaves the carbon–mercury bond is of fundamental interest for biological processing of organometallics.

Mechanistic Analysis. Electrophilic carbon–mercury bond cleavage reactions could, in principle, occur via one of four different pathways, involving either a carbocation, a carbanion, a radical, or a concerted process. Previous work in our laboratory has allowed us to distinguish among these possibilities.[23] A solvolysis reaction via an S_N1 (carbocation) pathway is highly unlikely on the basis of formation of hydrocarbons rather than alcohols from the enzymatic reaction. An S_E1 pathway can also be eliminated since none of the substrates that were studied are capable of stabilizing an intermediate carbanion. In addition, this pathway has only rarely been observed in the nonenzymatic reaction.[27-29] Of the last two alternatives, a homolytic radical cleavage does have chemical precedent in the protonolysis of diorganomercurials, but not conclusively in the case of organomercurial salts,[30] while a concerted S_E2 type cleavage has been demonstrated in kinetic and stereochemical studies in the protonolysis of vinyl mercurials, diorganomercurials, and alkyl mercurial salts.[24,31,32] Therefore, the stereochemically defined substrates described below were chosen to provide specific probes for radical rearrangements and to determine the stereochemical outcome of the carbon–mercury bond cleavage[23] (Figure 2).

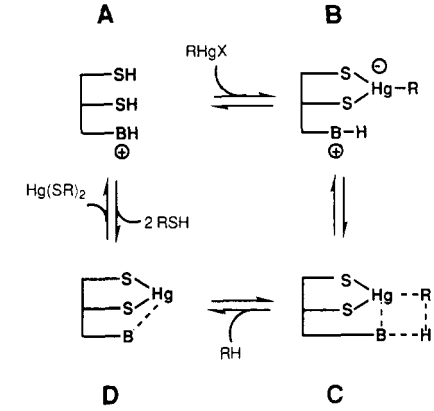

Figure 3. Proposed S_E2 mechanism for carbon–mercury bond cleavage by MerB. Given that all four cysteines in the *E. coli* R831 enzyme are required for catalysis, it is possible that BH^+ is a cysteine residue.

Radical Probes. Substrates **1** and **2** (Figure 2) were monitored for skeletal rearrangement to determine if the enzymatic reaction proceeded by a radical mechanism. These studies were based on the chemical precedent provided by Whitesides and San Filippo,[33] who showed that the borohydride-mediated reduction of these tricyclic and bicyclic mercurials yielded an equal mixture of the nortricyclene and norbornene product isomers. These results were taken to be strong evidence for rapidly equilibrating radical intermediates. In contrast, MerB reduced the same substrates to yield only the unrearranged hydrocarbon products to the limits of detection by GC (1%).

Stereochemical Probes. When the substrate *cis*-butenylmercuric chloride (**3**) was processed by the enzyme, the product was exclusively the *cis*-butene, with no detectable amount (<1%) of *trans*-butene present (which would have been expected if a radical intermediate was involved). Furthermore, when *endo*-2-norbornylmercuric bromide (**4**) was cleaved enzymatically in D_2O, giving [2-D]norbornane as product, the deuterium was located only in the endo position as monitored by FTIR. This high degree of retention of configuration seen in the enzymatic cleavage of these carbon–mercury bonds also argues against a radical-based mechanism and is consistent with a concerted S_E2 type mechanism.

Mechanistic Proposal. Our general mechanistic hypothesis for MerB is shown in Figure 3 and represents *the first proposal* of a biological S_E2 type mechanism.[23] In this mechanism, the organomercurial would first form an enzyme–substrate complex via initial binding to one or two nucleophilic residues (suggested to be cysteine SH groups) in the enzyme active site (B). Coordination by the nucleophilic residues would polarize and perhaps activate the carbon–mercury bond for protonolysis. X-ray crystallographic studies of organomercurials complexed with amino acids have documented such secondary interactions,[34-36] and nucleo-

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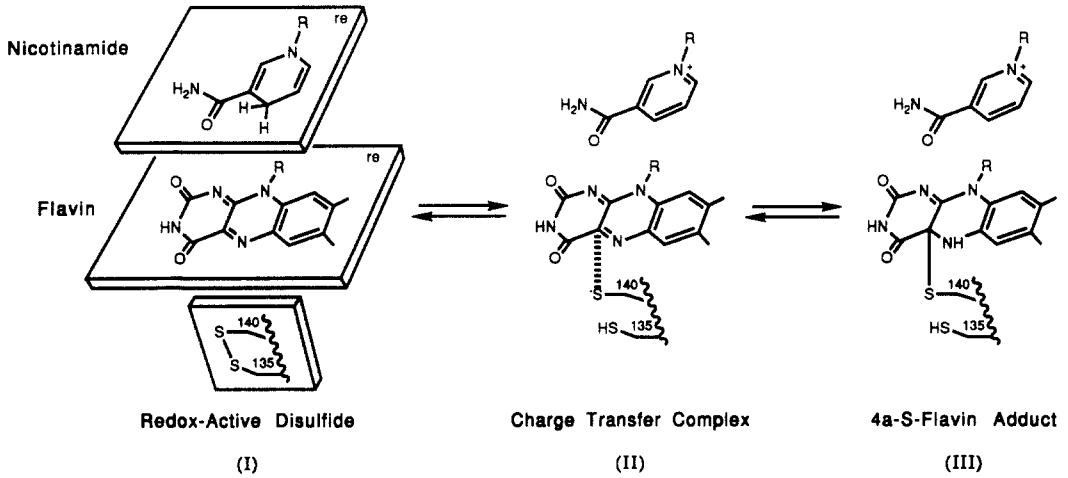

Figure 4. Stacked arrangement of cofactors in the active site of MerA and redox states of the redox-active disulfide.

philic accelerations of electrophilic reactions involving carbon–mercury bonds are known.[37] A hydrophobic pocket in the enzyme might further orient the very nonpolar organomercurial into the proper position by providing a lipophilic microenvironment.[23] The identity of the active-site moiety responsible for delivering the proton during catalysis (C) is under study and may also be a cysteine residue (vide infra). No evidence is available as to whether a tri- or a tetracoordinate substrate or product mercury complex forms during catalysis; however, there is recent evidence that the MerR protein supports a tricoordinate mercury complex.[38-40] Enzymatic cleavage of the carbon–mercury bond via an S_E2 mechanism would then yield the hydrocarbon product and the enzyme–[Hg(II)] complex (D). Finally, exchange of Hg(II) with exogenous thiols leads to free enzyme (A) ready for another round of catalysis.[23]

Molecular Biological Probes of MerB Structure and Function. To understand further the nature of the MerB catalyst requires knowledge about both the enzyme structure and the catalytic roles of specific amino acid residues. The *merB* genes from several bacterial sources have been sequenced,[15,41] including *merB* from *Escherichia coli* R831,[42] the source of the purified enzyme. *E. coli* MerB has four cysteines; three of these are highly conserved across the known sequences, making them likely candidates for residues ligating RHgX and Hg(II). Cysteine to alanine mutations have been engineered at positions 101, 122, 164, and 165. Each mutant enzyme is defective in conferring resistance to phenylmercuric acetate when assayed in vivo, suggesting that all four cysteines in the *E. coli* MerB are essential for catalysis.[43] Overproduction and purification of the mutant enzymes to assess their in vitro substrate binding and catalytic capacities are underway. Ultimately, knowledge of active site residue geometry and function may allow redesign of MerB to cleave other organometallics, such as toxic organolead compounds. The enzyme already possesss a weak ability to cleave certain organostannanes,[44] but that activity may not be optimized.

Mercuric Ion Reductase

Mercuric ion reductase (MerA) is an NADPH-dependent flavoprotein[45] that has an α_2-homodimeric native structure[46,47] containing two active sites per dimer. In addition to the flavin (FAD), each active site also contains a pair of cysteines (Cys_{135} Cys_{140} in Tn*501* MerA[11,48]) which form a so-called redox-active disulfide (Figure 4, I). This disulfide is in close proximity to the flavin isoalloxazine ring, allowing its rapid reduction by dihydroflavin, $FADH^-$, whereupon a charge-transfer complex (Figure 4, II) is formed between oxidized FAD and the Cys_{140} thiolate (pK_a 5.0[49]). These features are characteristic of the flavin-containing disulfide oxidoreductase class of enzymes, a family that includes glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase.[50,51] All members of this family share similar spectral properties (including the diagnostic flavin–thiolate charge-transfer band) and high active-site sequence homology. To date, the only disulfide oxidoreductase whose X-ray crystal structure has been solved to high resolution (1.5 Å) is human glutathione reductase;[52] presumably other family members have similar active-site alignments with the NAD(P)H, flavin, and redox-active cystine being in a stacked array (Figure 4, I).[53]

Though structurally similar to MerA, other disulfide oxidoreductases all catalyze the flow of electrons be-

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Table III
Relative Rates of Hg(II) Reduction Catalyzed by Wild-Type and Some Mutant Mercuric Reductases

enzyme	turnover no., min^{-1}	rel rate
wild type	≫340[a]	17 000
C135A	0.2[b]	10
C140A	0.03[b]	2
C135A C140A	0.02[b]	1
C558A	5[c]	250
C559A	120[c]	6000
C558A C559A	0.3[d]	15
yeast glutathione reductase	0.03[d]	2
dihydroflavin, $FADH^-$	0.3[b]	20

[a] k_{cat} for Hg(II) reduction in the presence of 1 mM 2-mercaptoethanol (37 °C). [b] Estimated from single-turnover reoxidation of enzyme-bound $FADH^-$ in the presence of 180 μM Hg(II)–EDTA (37 °C). See footnote *c*, Table I. [c] k_{cat} for Hg(II) reduction in the presence of 1 mM 2-mercaptoethanol (25 °C). [d] Rate of Hg(0) volatilization in the presence of 100 μM $HgCl_2$ and 1 mM cysteine (37 °C).

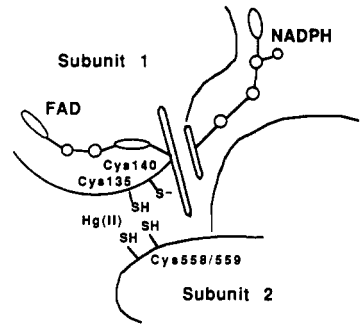

Figure 5. Proposed intersubunit active site structure of the EH_2–NADPH complex of MerA. This scheme was adapted from the known human glutathione reductase active site structure. It shows a four-cysteine-containing active site which lies at the interface between subunits in the MerA dimer. No specific chelation of Hg(II) is shown as its exact mode of binding during catalysis has not yet been determined (see text).

tween NAD(P)H and dithiol or disulfide substrates (and some peroxides[54,55]); they do not readily reduce metallic species. In fact, glutathione reductase and lipoamide dehydrogenase are known to be potently inhibited by mercurials, which bind to their active-site thiols.[56,57] Thus, one major objective of work in this and other laboratories over the past decade has been to delineate the unique structural and mechanistic features of MerA which allow it avoid similar inhibition and actually reduce Hg(II) to Hg(0) at a biologically useful rate. A second area of interest concerns the extreme substrate specificity exhibited by MerA, which catalyzes no significant oxidation or reduction of any metal species, other than Hg(II), so far tested.[47,58]

As one approach toward addressing the above issues, our laboratory has pursued site-directed mutagenesis to provide Tn*501* MerA mutants with altered catalytic properties.[59–61] In vivo and in vitro analysis of both wild-type and mutant MerA's has revealed many details about both mechanism and active-site organization. [It should be noted that this mutational analysis has been carried out in the absence of an X-ray crystal structure (vide infra). Nevertheless, by examining their UV–vis spectroscopic and flavin-mediated catalytic properties (e.g., thio$NADP^+$/NADPH transhydrogenation), we have been able to show that all the mutants discussed below have structurally intact sites in which electron transfer between NADPH and flavin is uncompromised. Thus, we feel confident that the observed alterations in catalytic activity for Hg(II) reduction are not due to gross structural changes, but rather reflect specific impairment of the Hg(II) to Hg(0) half reaction.]

Tn*501* MerA has eight cysteines, six of which are paired. One pair, Cys_{135} Cys_{140}, forms the redox-active disulfide, but the other two, Cys_{10} Cys_{13} and Cys_{558} Cys_{559}, are contained within sequences having no homology to other disulfide oxidoreductases.[11] We have explored the functions of these six residues by changing each in turn to serine or alanine. As expected, alteration of either or both of the redox-active thiols (Cys_{135} Cys_{140}) results in spectral changes and marked attenuation in catalytic activity.[59,60] In contrast, the N-terminal cysteine pair (Cys_{10} Cys_{13}) had been postulated to be involved in intracellular Hg(II) transport,[8,62] yet both residues can be changed to alanines without significantly affecting the in vivo mercury resistance phenotype.[61] Thus this pair is fully dispensable, and indeed a proteolytic fragment of the wild-type enzyme lacking residues 1–85 is wholly active in vitro.[48] Alteration of the C-terminal thiols (Cys_{558} Cys_{559}), however, results in partial to extreme attenuation of catalytic efficiency, with specific activities for Hg(II) reduction ranging from ca. one-third the wild-type level for the C559A[63] mutant to 1/1000 wild type for the C558A C559A double mutant (Table III).[61,64] Although the C-terminal cysteines do not affect the flavin absorbance spectrum, they do perturb its fluorescence, suggesting that the Cys_{558} Cys_{559} pair is near the chromophore, though not as close as Cys_{135} Cys_{140}. Further, the C-terminal cysteines are near enough to Cys_{135} Cys_{140} to be capable of dithiol/disulfide interchange.[65] Therefore, in addition to the conserved redox-active thiols, the active site of MerA contains a second pair of cysteines, located near the C-terminus, which are required for full catalytic activity. In fact, it is these unique C-terminal cysteines that are largely responsible for the ability of MerA to reduce Hg(II) at much higher rates than related oxidoreductases. This is dramatically illustrated by the observation that removal of both Cys_{558} and Cys_{559} from MerA (C558A C559A) results in a decrease in its Hg(II) reduction rate by 3 orders of magnitude, while the rest of the enzyme molecule contributes only a 10-fold rate increase over the adventitious Hg(II) reduction rate exhibited by glutathione reductase (Table III).[61]

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(63) A standard notation for site-directed mutations consists of the one-letter amino acid code and position of the wild-type residue followed by the one-letter amino acid code for the mutation; e.g., C559A indicates that the cysteine at position 559 has been changed to alanine.
(64) Moore, M. J.; Miller, S. M.; Walsh, C. T., to be published.
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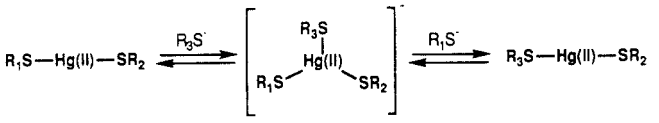
Scheme I



Though no X-ray structure yet exists (*Bacillus* RC607 MerA has only recently been crystallized[66]), we have used mixed mutant heterodimers to demonstrate that the two active-site cysteine pairs originate from separate subunits; i.e., when the C135A C140A and C558A C559A double mutants are combined to form mixed heterodimers in vivo or in vitro, the ability to reduce mercuric ions is restored.[67] This, coupled with the other results discussed above, has allowed us to propose an intersubunit active site as schematized in Figure 5. This model has been adapted from the known crystal structure of human glutathione reductase[68] in which the active site contains a redox-active disulfide, Cys_{58} Cys_{63}, from one subunit and an essential base, His_{467}, from the adjacent subunit.

Issues for Enzymatic Hg(II) Reduction. In considering the actual chemistry involved in reduction of Hg(II) by MerA, two central questions emerge: (1) what is the relevant ligation sphere of Hg(II) within the enzyme active site, and (2) what is the actual mechanism of electron transfer? The magnitude of the first problem can be appreciated by examination of the thermodynamics of Hg(II) reduction. First, the reduction potentials for free Hg(II) and NADPH, 850 mV and −320 mV, respectively, indicate that the overall reaction catalyzed by MerA is thermodynamically very favorable. However, the tendency of Hg(II) to form very stable $Hg(SR)_n$ complexes (vide infra) dramatically decreases its reduction potential (e.g., the midpoint potential of $Hg(Cys)_2$ is −270 mV,[69] while those for bis-thiol chelates have been calculated to be as low as −390 to −475 mV[65]). Therefore, enzymatic transfer of electrons from NADPH to Hg(II) chelated within the active site may be thermodynamically unfavorable.

It is clear that MerA faces a dilemma: it must provide an excellent binding site for Hg(II) capable of outcompeting other cellular ligands, but it must do so without making reduction to Hg(0) too unfavorable. How might the enzyme overcome this apparent difficulty? Initial velocity measurements with wild-type MerA have demonstrated that all four active-site cysteines must be reduced to thiols before catalytic Hg(II) reduction can occur.[70] To account for this multiple thiol requirement, Miller et al.[65] proposed that MerA adopts a tri- or tetradentate Hg(II) chelate as the reactive species (although our more recent observation of high activity levels with the C559A mutant[64] would seem to rule out the latter). Though less stable than bidentate complexes [e.g., $K_{diss} \sim 10^{-3}$ M for Hg(glutathione)$_3$ as compared to 10^{-40} M^2 for Hg(glutathione)$_2$[71]], both tri- and tetracoordinate mononuclear

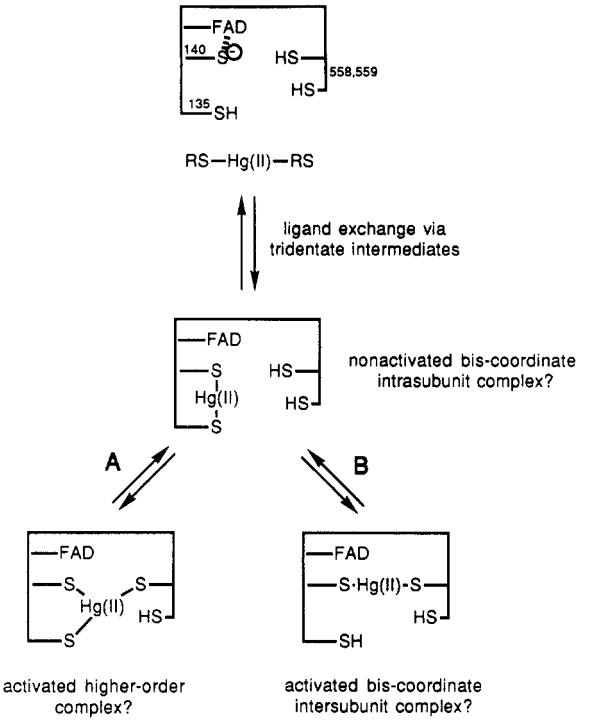


Figure 6. Possible modes of ligation of Hg(II) within the active site of MerA.

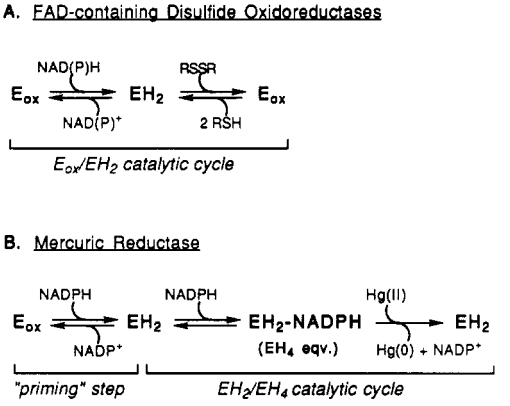


Figure 7. Minimal catalytic mechanisms for the disulfide oxidoreductases and MerA showing their respective redox cycles.

Hg(II)–thiol complexes have been observed in the solid state,[72,73] and in solution $Hg(SR)_2$ complexes undergo rapid ligand exchange via a tridentate intermediate (Scheme I).[71] Considering the high effective concentration of the four thiols within the active site of MerA, the enzyme surely utilizes multiple thiols to receive and correctly position Hg(II) in its active site, therein achieving at least transient tridentate coordination (Figure 6).

While the capacity for higher order coordination likely contributes to the overall binding affinity of MerA for Hg(II), the question of whether it is required during the actual reduction step remains the subject of much controversy. Miller et al.[65] have argued that it is (Figure 6, pathway A), reasoning that the greater electronic repulsion introduced by additional thiol ligands around

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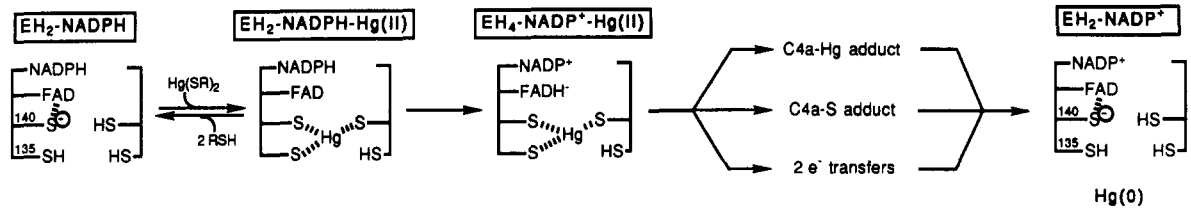

Figure 8. Possible mechanisms for reduction of Hg(II) by $FADH^-$ in the active site of MerA. Dashed lines between Hg(II) and thiols signify an undetermined mode of ligation. Three of the four active-site cysteines are shown as putative Hg(II) ligands since mutation of any of the three leads to substantial drops (<2% residual activity; Table III) in catalytic efficiency.

Hg(II) may raise the ground-state energy of such a complex relative to biscoordinate Hg(II), thereby effectively lowering the activation energy for reduction and increasing the rate of reaction. However, recent model studies with a putative tetrahedral four-coordinate Hg(II) complex have implied that increasing the number of thiols chelated to Hg(II) only results in a more negative reduction potential.[72] From this thermodynamic result, Bruice and co-workers concluded that higher thiol coordination cannot facilitate enzyme catalysis. A relevant issue is whether tri- or tetracoordinate ligation occurs in the enzyme active site in such a way as to generate a ligand sphere with distorted geometry and distance. Such complexation, in addition to the entropic advantage of holding Hg(II) in proximity to the electron-rich $FADH^-$ (vide infra), might result in a net catalytic advantage for Hg(II) reduction. It should be noted that a recent EXAFS study of wild-type and C558A C559A MerA's failed to detect any significant accumulation of tri- or tetracoordinate Hg(II) bound in the active site,[73] so if such species are formed they must be transient or present at low concentrations. Instead the EXAFS results clearly showed that the bulk species in both the mutant and wild type was a bis-thiol Hg(II) chelate.

Another model that would account for the multiple thiol requirement and not invoke higher order chelates is shown in Figure 6, pathway B. In this model, the reductive pathway utilized by MerA would require an intersubunit biscoordinate conformer, such as Cys_{140}–Hg(II)–Cys_{558}. The inabilities of the C558A C559A double mutant and other disulfide oxidoreductases to catalyze Hg(II) reduction at significant rates (vide supra) and of wild-type MerA to reduce other metal ions suggest that Hg(II) reduction may be highly sensitive to electronic and/or spatial constraints (note that, on the basis of the glutathione reductase crystal structure, a linear bidentate redox-active dithiol mercury chelate would be perpendicular to the flavin). An intersubunit chelate might overcome these barriers by changing both the $FADH^-$–Hg(II) distance and the orientation of the Hg(II) complex with respect to the isoalloxazine ring. We are currently constructing new double-mutant enzymes (e.g., C135A C559A) in order to test this hypothesis.

With regard to the question of exactly how electrons are transferred from NADPH to Hg(II), MerA again differs from its disulfide oxidoreductase homologues. The latter enzymes all cycle between their FAD_{ox}, redox-active disulfide (E_{ox}) and FAD_{ox}, redox-active dithiol (EH_2) forms, coupled with dithiol/disulfide interchange between the substrate and the redox-active dithiols (Figure 7A). In these enzymes, the flavin serves merely as a conduit of electrons between NAD(P)H and the redox-active disulfide. In contrast, single turnover experiments have shown that while the EH_2 state of MerA can bind Hg(II), it is not catalytically competent to reduce it.[70] Instead, this enzyme requires a four-electron (rather than two-electron) redox inventory for catalysis. MerA cycles between its two- and four-electron-reduced states,[76] with the predominant four-electron-reduced species being EH_2–NADPH[77] (Figure 7B). EH_4–$NADP^+$ ($FADH^-$, redox-active dithiol) was not detected in wild-type MerA under turnover conditions;[78] presumably the charge-transfer interaction between the Cys_{140} thiolate and FAD_{ox} stabilizes the EH_2–NADPH redox state, thus preventing discernible accumulation of the EH_4–$NADP^+$ species.[79] Even so, it would appear that MerA does utilize dihydroflavin, $FADH^-$, rather than the redox-active dithiol, as the actual reductant for Hg(II). This significant departure from other disulfide oxidoreductase mechanisms is supported by two key observations: (1) mutants in Cys_{135} are viable for Hg(II) reduction through multiple turnovers[58,60] even though they do not have the capacity to form a redox-active disulfide (Table III), and (2) dihydroflavin free in solution can reduce Hg(II) to Hg(0).[58,60,74] In the wild-type enzyme it seems likely that upon Hg(II) ligation to Cys_{140}, which disrupts the charge-transfer complex, reduction of FAD by NADPH becomes more favorable, facilitating formation of EH_4–$NADP^+$ in which $FADH^-$ functions as the kinetically competent reductant (Figure 8).

The unique catalytic ability of MerA then devolves to the chemical mechanism by which dihydroflavin actually reduces Hg(II) to Hg(0). However, the transient nature of any $FADH^-$ and $Hg(Cys)_3$ species occurring within the active site (vide supra), along with the general rapidity of the electron-transfer reactions ($>10^6$ s^{-1}),[80] makes experimental determination of the precise electron-transfer mechanism very difficult. At the present time we cannot differentiate between the three possible mechanisms outlined in Figure 8.[65] The first involves nucleophilic attack of the flavin C-4a on the electropositive Hg(II), followed by elimination of Hg(0). Precedence for this addition–elimination path-

(76) Note that the two-electron (EH_2) and four-electron (EH_4) redox states refer only to the electron content of the redox-active disulfide and FAD. While the presence of the C-terminal cysteine pair formally gives MerA a six-electron redox capacity, Cys_{558} and Cys_{559} remain thiols under physiological conditions; i.e., they do not normally undergo reversible oxidation and reduction (see ref 65).

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(79) However, there is kinetic evidence that EH_4–$NADP^+$ is present at small concentrations under turnover conditions. This comes from the observation that EHR (Cys_{135}–SCH_2CONH_2, Cys_{140}–S^- wild-type enzyme) catalyzes both thio$NADP^+$ transhydrogenation and O_2 reduction (to H_2O_2), processes that are necessarily mediated by an $FADH^-$ species in this modified enzyme since EHR contains no functional redox-active disulfide (see refs 48 and 60).

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way lies in its similarity to the allylic oxidation of olefins by Hg(II)[81] and to the mercuration of $FADH^-$-related heterocyclic ring systems.[82] The second mechanism invokes nucleophilic attack of $FADH^-$ on the Cys_{140} thiolate ligand attached to Hg(II), resulting in an outer-sphere reduction of the metal to Hg(0) with concomitant formation of an intermediate flavin C-4a thiol adduct. While no direct chemical precedent exists for such an outer-sphere reduction of Hg(II), mechanisms utilizing a "bridging" sulfur ligand (analogous to the Cys_{140} thiolate) are well-known in the redox reactions between Co(III) and Cr(II).[83] Also, strong evidence has recently been obtained that a C-4a Cys_{140} adduct can readily form in MerA (Figure 4, III).[84] The third possible enzyme mechanism is that of two successive single-electron transfers from dihydroflavin to Hg(II), with Hg(I) as an intermediate, similar to that previously implicated in the reduction of Hg(II) by flavin semiquinone.[85] A fourth possibility not shown is that of a formal two-electron "hydride" transfer from $FADH^-$ to Hg(II), similar to the reduction of Hg(II) by metal hydride reagents.[33] However, this mechanism seems highly unlikely in light of our findings that 5-deaza-$FADH_2$, either free in solution[58,60] or bound within the enzyme active site,[86] cannot reduce Hg(II).

Conclusions. In this Account we have described two unique enzymes, organomercurial lyase (MerB) and mercuric ion reductase (MerA), that have evolved efficient strategies to carry out organometallic and bioinorganic chemistry on mercury species. Study of the structure and function of these enzymes has revealed the importance of key cysteinyl thiols for the ligation of RHgX and Hg(II) in such a way as to selectively lower the energy barriers for protonolytic fragmentation of C–Hg bonds (MerB) and electron transfer to thiol-coordinated Hg(II) species (MerA). Understanding of the catalytic mechanisms of these enzymes may ultimately allow purposeful redesign of their genes and encoded proteins to permit enzymatic processing of other organometallic and inorganic compounds in the environment.

We acknowledge the significant contributions to the MerA and MerB projects made in this laboratory by Drs. Barbara Fox, Karin Au, Peter Schultz, Tadhg Begley, and Alan Waltz. Additionally, many of the experiments described in the MerA section were performed in collaboration with Drs. Susan Miller, Vincent Massey, David Ballou, and Charles Williams, Jr., at the University of Michigan; current understanding of the MerA mechanism is due in large part their input and expertise. Nikolaus Schiering and Dr. Emil Pai at the Max Planck Institut für Medizinische Forschung, Heidelberg, FRG, continue efforts to solve the MerA crystal structure.

Registry No. Hg, 7439-97-6; mercuric ion reductase, 67880-93-7; organomercurial lyase, 72560-99-7.

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Mechanistic and Evolutionary Aspects of Vitamin B_{12} Biosynthesis

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Introduction

Since the discovery of vitamin B_{12} as the anti-pernicious anemia factor, chemists and biochemists alike have been fascinated not only by the complex problems inherent in the structure of B_{12}, the biochemistry mediated by its coenzyme, and the total synthesis of the corrin macrocycle but also by the challenge of unraveling the biosynthetic pathway, which is shared at the outset by nature's route to the other biologically important metallopigments—heme and siroheme (iron), chlorophyll (magnesium), and coenzyme F430 (nickel)—before diverging along the corrin (cobalt) branch.

For over 20 years we and others have been studying the B_{12} pathway, the first decade of which was reviewed in earlier Accounts.[1] The results of these endeavors are summarized in Scheme I, which introduces the early intermediates 5-aminolevulinic acid (ALA) and por-

A. Ian Scott was born in Glasgow, Scotland, in 1928. After receiving B.Sc., Ph.D., and D.Sc. degrees from Glasgow University (with R. A. Raphael), he spent a postdoctoral year with M. S. Newman (Ohio State) and, following an industrial position (Imperial Chemical Industries), joined Sir Derek Barton's laboratory for a second postdoctoral period in London and Glasgow. His academic career began in 1957 at Glasgow and was followed by Professorships at U.B.C. (Vancouver), Sussex (U.K.), Yale, and finally Texas A&M University, where he has remained, apart from a brief return to Scotland in 1980–1981, since 1977. He is a Fellow of the Royal Societies of London and Edinburgh and a founding member of the Yale Sherlock Holmes Society. His research interests have been focused for the last 20 years on the biosynthesis of porphyrins and B_{12} and on enzyme mechanisms, investigated by using a combination of NMR spectroscopy, organic chemistry and, more recently, molecular biology.

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