Crystal Structures of the Organomercurial Lyase MerB in Its Free and Mercury-bound Forms

INSIGHTS INTO THE MECHANISM OF METHYLMERCURY DEGRADATION

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Bacteria resistant to methylmercury utilize two enzymes (MerA and MerB) to degrade methylmercury to the less toxic elemental mercury. The crucial step is the cleavage of the carbon-mercury bond of methymercury by the organomercurial lyase (MerB). In this study, we determined high resolution crystal structures of MerB in both the free (1.76 Å resolution) and mercury-bound (1.64 Å resolution) states. The crystal structure of free MerB is very similar to the NMR structure, but important differences are observed when comparing the two structures. In the crystal structure, an amino-terminal α-helix that is not present in the NMR structure makes contact with the core region adjacent to the catalytic site. This interaction between the amino-terminal helix and the core serves to bury the active site of MerB. The crystal structures also provide detailed insights into the mechanism of carbon-mercury bond cleavage by MerB. The structures demonstrate that two conserved cysteines (Cys-96 and Cys-159) play a role in substrate binding, carbon-mercury bond cleavage, and controlled product (ionic mercury) release. In addition, the structures establish that an aspartic acid (Asp-99) in the active site plays a crucial role in the proton transfer step required for the cleavage of the carbon-mercury bond. These findings are an important step in understanding the mechanism of carbon-mercury bond cleavage by MerB.

In the environment, mercury can exist in three different forms: elemental mercury, ionic mercury, and methylmercury. The toxicity of methylmercury is attributed to its lipophilicity and affinity for thiol residues found in proteins (1). The ability of methylmercury to accumulate in fat tissues leads to its biomagnification within the food chain and ultimately to its toxicity (2, 3). An example of methylmercury bioaccumulation is the high levels found in fish species in contaminated waterways. Select fish species accumulate high levels of methylmercury, and consumption of these contaminated species poses a serious health threat to humans.

Because of the high number of mercury-contaminated waterways throughout the world, there is a need to develop an effective remediation system for methylmercury. Most systems for cleanup of mercury-contaminated sediments use either physical or chemical remediation. Unfortunately these systems are expensive and environmentally disruptive. In the 1960s, bacteria were isolated from soils and sediments contaminated with high levels of mercury (4–6). Analysis of these bacteria demonstrated that they had acquired a series of plasmid-encoded genes collectively referred to as the mer operon that imparts resistance to mercury. Although the precise composition of the mer operon varies between bacterial strains, strains resistant to high levels of methylmercury code for two enzymes (MerA and MerB) that transform methylmercury to elemental mercury (7). The organomercurial lyase MerB cleaves the carbon-mercury bond of methymercury, releases methane (8), and directly transfers the ionic mercury to the mercurial reductase MerA. MerA reduces the ionic mercury to elemental mercury, which is volatile and diffuses out of the bacteria (9). Because of the unique properties of these two enzymes, there are numerous ongoing attempts to exploit them in bioremediation systems to clean up methylmercury contamination (10–13).

MerB cleaves the carbon-mercury bond of many organomercurials to generate ionic mercury and a protonated carbon species (14). Based on kinetic experiments, it was concluded that the reaction mechanism is a bimolecular electrophilic substitution (S 2') (15). In this mechanism, a proton attacks the carbon moiety from the same side as the mercury, and bond cleavage and protonation occur with retention of the stereochemistry at the carbon position. Based on subsequent mutagenesis experiments, more detailed models have been proposed describing the catalytic role of cysteine residues (16). Mutagenesis studies with MerB from Escherichia coli (plasmid R831b) demonstrated that two highly conserved cysteines (Cys-96 and Cys-159) were essential for catalytic activity. The NMR structure of free MerB confirmed that these two cysteines are in close proximity within the active site (17). Recently model compounds have been shown to cleave carbon-mercury bonds using only...
thiol groups, and their unique properties have been proposed to mimic the mechanism of MerB and specifically Cys-96 and Cys-159 (18). Despite these efforts, the precise details of the mechanism by which MerB cleaves methylmercury are still poorly understood.

In this study, we determined high resolution crystal structures of MerB in the free (1.76-Å resolution) and mercury-bound (1.64-Å resolution) states. The two structures of MerB provide important insights into the catalytic mechanism of this unique enzyme. The structures support earlier studies indicating that Cys-96 and Cys-159 are important for the binding to organomercurials and suggest that Asp-99 is an active site residue that participates in the protonolysis of the carbon-mercury bond.

**MATERIALS AND METHODS**

Expression of Recombinant Proteins—The sequence encoding MerB from *E. coli* plasmid R831b was cloned as described previously (17). The MerB mutants (C96S MerB, C159S MerB, and C160S MerB) were prepared by site-directed mutagenesis of plasmid pQZB1 (16). Wild-type MerB was expressed and purified as described previously (16, 17). Prior to crystallization, proteins were dialyzed (see supplemental methods).

Crystalization—Crystals of MerB, C96S MerB, C159S MerB, and C160S MerB were grown by vapor diffusion at 23 °C using either a 1:1 or 1:2 mixture of protein solution (8 mg/ml initial protein concentration) and precipitant buffer, respectively, that was equilibrated against a reservoir of precipitant buffer (see supplemental methods).

Data Collection and Processing—MerB crystals were soaked for precise times (7, 10, and 25 min) in organomercurial buffer (precipitant buffer plus a 1.0 mM concentration of either methylmercury or para-hydroxymercury benzoic acid plus 10 mM 1,4-DTTB or cysteine). Diffraction data were collected from single crystals at beam line X29 of the National Synchrotron Light Source (Brookhaven National Laboratory, Upton, NY) using an ADSC Quantum-315 charge-coupled device (Area Detector Systems, Poway, CA), beam line X12b of the National Synchrotron Light Source (Brookhaven National Laboratory) using an ADSC Quantum-4 charge-coupled device (Area Detector Systems), or beam line 22-BM of the Advanced Photon Source (Argonne National Laboratory, Argonne, IL) using an MX-225 system (Rayon/Max USA, Evanston, IL). All data sets were processed with HKL2000 (19), and the results are summarized in Table 1 and in supplemental Table 2.

**RESULTS**

Crystalization Conditions and Structure Determination—MerB crystals were obtained at pH 5.5 and then soaked with several different organomercurial analogs including methylmercury to generate the mercury-bound MerB. The MerB crystal structure is a homodimer in the asymmetric unit, although MerB in solution has been shown to be monomeric (Fig. 1) (14, 17). A 2-fold non-crystallographic symmetry exists between the two subunits (A and B) present in the crystal. MerB from *E. coli* (R831b) is 212 amino acids long, and residues 1–208 were used to refine subunit A. For subunit B, residues 1–204 were used with the exception of residues 13–18 and 149–151. The average B-factor for C₅ atoms is 19.5 Å² for the two monomers, 17.4 Å² for subunit A, and 21.8 Å² for subunit B. Despite differences in the data quality, the two subunits have identical folds.

Comparison between the Crystal and NMR Structures of Free MerB—The NMR structure demonstrated that MerB consists of three antiparallel β-sheets surrounded by six α-helices that

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**TABLE 1**

Data collection and structural refinement statistics for MerB in the free and mercury-bound forms

| Soaking experiment details | Free form | Mercury-bound form |
|-----------------------------|-----------|--------------------|
| Organomercurial            | 1 mM PHMBAa |
| Exogenous thiol            | 10 mM L-Cys |
| Time (min)                 | 10         | 10                 |

| Data collection statistics | Free form | Mercury-bound form |
|----------------------------|-----------|--------------------|
| Space group                | P2₁       | P2₁                |
| Unit cell parameters       |           |                    |
| a (Å), b (Å), c (Å)        | 38.7, 90.0, 52.2 | 38.4, 89.2, 51.6   |
| β (°)                      | 100.5     | 100.5              |
| Resolution (Å)             | 20-1.76   | 20-1.64 (1.70-1.64) |

| Table 1                  |           |                    |
|--------------------------|-----------|--------------------|
| Unique reflections        | 33,974    | 40,293             |
| Completeness (%)          | 97.9 (90.9) | 96.6 (93.2)       |
| Rmerge (%)                | 0.050 (0.181) | 0.064 (0.481)   |
| Rp(e,λ) (%)               | 25.7 (6.3) | 17.7 (2.5)       |
| Rfree (%)                 | 3.7 (2.6) | 3.9 (3.5)       |

| Model refinement statistics | Free form | Mercury-bound form |
|-----------------------------|-----------|--------------------|
| No. of reflections          | 33,441    | 39,084             |
| R cryst (%)                 | 18.2      | 18.5               |
| R cryst (%)                 | 20.8      | 21.3               |
| Protein atoms               | 3,084     | 3,084              |
| Water molecules             | 347       | 311                |
| Average B-factor (Å²)       | 21.6      | 21.9               |
| Estimated coordinate error  | 0.25      | 0.18               |
| r.m.s. deviations Bonds (%) | 0.005     | 0.006              |
| Angles (°)                  | 1.974     | 1.049              |
| Protein Data Bank code      | 3FOO      | 3FOP               |

a para-Hydroxymercury benzoic acid.

b Values in parentheses correspond to the highest resolution shell.

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The abbreviations used are: DTT, dithiothreitol; [TrnH]K, tris[2-mercaptop-1-t-butylimidazolyl]hydroborate.
Crystal Structure of MerB

FIGURE 1. Crystal structure of free MerB at 1.76-Å resolution. MerB forms a dimer in the asymmetric unit related by a 2-fold pseudosymmetry as depicted by the rotation axis. The color of the two subunits varies from blue to green starting from the amino-terminal end of the protein to help distinguish the amino-terminal end of the protein from the carboxy-terminal end of the protein. Active site residues are highlighted with a colored van der Waals sphere representation: residues Cys-96 and Cys-159 are in yellow, and residue Asp-99 is in red.

FIGURE 2. Stereoview of the alignment of the crystal structure of MerB (blue) on its NMR structure (pink). The figure highlights the differences of the amino-terminal helix (residues 3–14) and the core domain (residues 76–200) in the two structures.

could be divided into two regions, an amino-terminal region and a core region. The crystal structure of free MerB is very similar to the solution structure (Fig. 2). Alignment of the backbone from the amino-terminal region and the core region of the two structures demonstrates that there are only minor differences (supplemental Fig. 5). However, comparison of the overall topology of the two structures demonstrates a number of important differences. The most striking differences are observed in the first 25 amino acids at the amino terminus and in a stretch of 8 amino acids (residues 154–161) located adjacent to the catalytic site. In addition, the crystal structure gave a more complete description of other regions of MerB (residues 73–82 and residues 95–104) that were not completely defined by the NMR data. In the solution structure, the first 25 amino acids at the amino terminus are not defined because of the lack of resonance assignments. In the crystal structures, residues 3–14 form an α-helix that is packed against the core region of MerB. This results in the entire amino-terminal region having a different orientation relative to the core region when compared with the NMR structure (Fig. 2). In addition, a new α-helix has been identified between residues 154–161 in the crystal structure that is absent in the NMR structure. The new helix is contained within a large flexible loop between residues Val-146 and His-161 in the NMR structure. In contrast, these residues are all well defined in the crystal structure with the exception of residues 149–151 in subunit B.

The differences between the two structures can be explained by factors that stabilize structural elements between the amino-terminal region (residues 3–14) and the core region (residues 146–161). The amino-terminal α-helix (residues 3–14) is interacting with the surface of the core region through van der Waals interactions involving Leu-3, Tyr-6, Ile-7, Leu-10, Leu-11, and Thr-17 as well as hydrogen bonds from the side chains of Tyr-6, Ser-13, and Ala-16. Residues 148–161 in the core region are thus stabilized near the active site by interactions with the amino terminus, and this helps explain the formation of the two helices that were not seen in the solution structure. In addition, the interaction between the amino-terminal helix and the flexible loop is stabilized by interactions between the two subunits.

Searches of the DALI structural data base with the MerB crystal coordinates failed to identify any new significant matches (31). The only protein with structural homology is the copper chaperone protein NosL (32), and the homology is only with the core region of MerB (Z = 3.5, root mean square deviation = 3.0 Å, calculated with DaliLite (33)).

The Active Site Is Buried in the Crystal Structure—MerB encoded by E. coli (R831b) contains four cysteines (Cys-96, Cys-117, Cys-159, and Cys-160), and NMR studies demonstrated that these cysteines (Cys-96, Cys-159, and Cys-160) are positioned near the active site (17). Point mutations demonstrated that the highly conserved Cys-96 and Cys-159 are essential for enzymatic activity, but replacing the non-conserved Cys-160 with a serine only moderately reduces activity (16). In the crystal structure, the sulfhydryl groups of Cys-96 and Cys-159 are buried within the protein core. They are buried as a result of the amino-terminal α-helix being in contact with the core region including the large loop between residues 148 and 161. There are two possible ways to access the sulfhydryl groups of Cys-96 and Cys-159 (Fig. 3). The first is by removing the loop (residues 148–161), and the second is by removing the amino-terminal α-helix. In contrast, Cys-160 is solvent-accessible (17).

Structure of Mercury-bound Form of MerB—To obtain a substrate-bound complex, crystals of free MerB were soaked with organomercurials (methylmercury or para-hydroxymercury benzoic acid) in the presence or absence of small exogenous thiols including L-DTT, cysteine, and glutathione. These experiments consistently generated a mercury-bound as opposed to an organomercurial-bound form of MerB. In every condition, there was no indication that either the carbon side chain or the exogenous thiol remained attached to the mercury (Fig. 4, A and B). This is in contrast to NMR studies where incubation of MerB with organomercurial compounds in the presence of DTT resulted in a MerB-Hg-DTT complex (34). The structure of mercury-bound MerB is very similar to that of the free MerB with the exception of the mercury ion, and the two structures
have a root mean square deviation difference of 0.190 Å about C_{\text{root}}. The mercury is bound to MerB in a planar-trigonal conformation by two sulfurs from Cys-96 and Cys-159 (at 2.3- and 2.4-Å distance, respectively), making a 127° angle. The third atom binding the mercury is oxygen from a bound water molecule at 2.6 Å, making angles of 122° and 111° with the mercury and the sulfurs of Cys-96 and Cys-159, respectively. As with the free MerB, the active site residues are completely inaccessible to solvent.

Crystal Structures of Cysteine Mutants—Given their importance, cysteine to serine mutants (C96S, C159S, and C160S) of MerB were prepared in an attempt to generate substrate-bound complexes. The structures of the three mutants were essentially identical to that of the native MerB in the free form. Following incubation with organomercurial compounds, we observed no evidence for bound organomercurial substrate or bound mercury product with either the C96S or C159S mutant (data not shown). In contrast, we observed bound mercury ion product in the C160S mutant (supplemental Table 2). This indicates that the C160S crystal retains enzymatic activity and that Cys-160 is not absolutely required for MerB activity. These results are in agreement with previous mutagenesis studies examining the effect of these mutants on MerB activity (16). In addition, these results indicate that Cys-96 and Cys-159 are essential for both substrate binding and for carbon-mercury bond cleavage.

Asp-99 Is in Position to Act as a Proton Mediator—As part of the mechanism of MerB, Cys-96 and/or Cys-159 must first be deprotonated and then react with the organomercurial compound to form a higher order structure about the mercury ion that makes the carbon-mercury bond more amenable to protonolysis. Analysis of the crystal structure of the mercury-bound form of MerB indicates that Asp-99 is the only amino acid vicinal to the bound mercury ion that can serve as proton mediator with one of its carboxylic oxygens within 2.9 Å and the second oxygen of the side chain within hydrogen bonding distance of the mercury.
the bound water attached to mercury. In addition, Asp-99 is in close proximity to the sulphydryl group of Cys-96 (3.6 Å) in the crystal structure of the free form and would be in an ideal position to function in the deprotonation step required for the sulphydryl group prior to attack of the organomercurial substrate.

**MerB and Chemicals Cleave by Similar Mechanisms**—It has recently been demonstrated that alkyl mercury compounds designed to mimic MerB are able to cleave organomercurial compounds (18, 35). One compound, tris(2-mercapto-1-t-buty1imidazoyl)hydroborate ([TmBu]K), forms a four-coordinated pseudotetrahedral mercury center containing three sulfurs and one carbon group ([TmBu]HgCH2CN) when reacted with ethyl mercuric chloride and potassium borohydride in acetonitrile (supplemental Fig. 6). Overlaying the crystal structures of [TmBu]HgCH2CN and the mercury-bound form of MerB about the mercury ion clearly demonstrates that Cys-96 and Cys-159 can be aligned with two or three sulfur atoms from [TmBu]HgCH2CN (Fig. 4C). This is despite the fact that mercury-bound form of MerB is in a trigonal geometry and the [TmBu]HgCH2CN is in a pseudotetrahedral geometry. In this overlay, the third sulfur atom of [TmBu]HgCH2CN is positioned in virtually the identical location as the carboxylate group of Asp-99, and the carbon of the uncleaved carbon-mercury bond of [TmBu]HgCH2CN is 3.1 Å away from one of the oxygen atoms of Asp-99. Given the facile nature with which [TmBu]HgCH2CN cleaves carbon-mercury bonds, this similarity provides additional evidence for the role of Asp-99 in the protonolysis of carbon-mercury bonds by MerB.

**DISCUSSION**

**Buried Active Site**—One important observation from the MerB crystal structures is the occurrence of the active site buried within the hydrophobic core. Burying the MerB active site could serve to inhibit the release of the product ionic mercury. Ionic mercury is highly toxic, and rapid release of mercury ion from MerB following cleavage of the carbon-mercury bond could result in extensive cellular damage. Retaining ionic mercury would enable MerB to transfer ionic mercury directly to the reductase MerA for conversion to the less toxic elemental mercury. Previous in vitro studies demonstrated that MerB shuttles ionic mercury to MerA, and the buried active site is consistent with this mechanism (34). MerA would compete with MerB for the mercury ion, and this would result in the direct transfer without release.

Two questions still remain. 1) How does the substrate enter the active site, and 2) how is mercury efficiently transferred to MerA? Based on our NMR studies, residues 146–161 are part of a flexible loop, and the 25 amino-terminal residues either exchange too rapidly or they are flexible. In the crystal structures, the high B-factors and the poor density of several residues in these regions support the possibility that these two regions of MerB are flexible and dynamic. Because Cys-159 is part of the flexible loop, substrate binding could trigger the stabilization of these two regions through a mutual interaction. The result of these interactions would be to bury the active site. This requires significant movements of structural elements and would result in a significant loss of entropy. The loss of entropy would reduce the overall activity of the enzyme, but the flexible active site enables MerB to cleave a wide range of organomercurial substrates. This rearrangement of structural elements following substrate binding is not only consistent with the low $k_{cat}$ (0.7–240 min$^{-1}$) and high $K_m$ values (0.5 mM for methylmercury) observed for MerB but also with the wide range of organomercurial substrates (methylmercury to para-hydroxymercury benzoic acid) that can be cleaved.

The geometry of the mercury in the mercury-bound MerB crystal is similar to what we previously observed for a MerB-Hg-DTT complex in solution (34). In this complex, the mercury ion binds Cys-96 and two sulfurs from DTT. In this structure, Cys-159 is in competition with the DTT for binding to the mercury. Given that the DTT is in excess, the Cys-159 sulphydryl can be displaced by the DTT. Despite this difference, there is a similarity of the preferred geometry (planar trigonal), and the distance (2.42 Å) of the three sulfurs to the mercury is approximately the same. This MerB-Hg-DTT intermediate can be viewed as one step further along the reaction pathway. The MerB-Hg-DTT complex demonstrates how a molecule with two sulphydryls can compete with Cys-159 for the mercury ion and serves as a model for how two cysteines of MerA could function to abstract the mercury ion from MerB in a shuttling mechanism (direct transfer).

**Role of Cysteines Residues**—The crystal structure of the cysteine mutants of MerB allowed us to analyze the roles for the three cysteines residues near the active site. The wild-type MerB crystals are enzymatically active, and in the presence of organomercurials a mercury-bound form is generated. In the mercury-bound form, Cys-96 and Cys-159 are bound to the mercury ion in a planar-trigonal geometry, and this form can be generated either in the presence or absence of exogenous thiols. In contrast, we were unable to obtain a mercury-bound form with crystals of either the C96S or C159S mutants, but we were able to generate a mercury-bound form with crystals of the C160S mutant. These results demonstrate that Cys-96 and Cys-159 have a role in substrate binding, carbon-mercury bond cleavage, and regulation of mercury ion release from the active site.

**Asp-99 as a Proton Donor**—The common mechanism for chemical cleavage of carbon-mercury bonds involves the activation of the mercury atom by nucleophilic ligands followed by protonolysis of the carbon-mercury bond (18, 35). Although difficult, the critical step appears to be the formation of organomercurials with high coordination numbers. Once the high coordination number has been achieved, the protonolysis step is relatively easy to perform (18). Comparison of the crystal structure of [TmBu]HgCH2CN with the crystal structures of MerB indicates that MerB cleaves the carbon-mercury bond in a manner similar to these chemical methods. As proposed earlier, Cys-96 would initially attack the organomercurial, and we postulated that the Asp-99 would deprotonate Cys-96. The carboxylate of Asp-99 is in closer proximity to Cys-96 (3.6 Å) in the free form than it is to Cys-159 (4.3 Å). Following attack by Cys-159 to form an organomercurial compound with a high coordination number, Asp-99 is perfectly positioned to donate the proton required for the protonolysis step. Thus, Asp-99 would serve as the proton mediator or acid/base catalyst by deprotonating the sulphydryl group of Cys-96.
In this study, we solved the crystal structure of MerB in its free and mercury-bound forms. These structures provide important information that is essential to ongoing attempts to exploit the potential of the mer system in the bioremediation of methylmercury.

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