The Roles of Thiols in the Bacterial Organomercurial Lyase (MerB)†

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

Bacterial resistance to mercury compounds occurs in two forms: narrow spectrum [resistance only to inorganic mercury, Hg(II)[1]] and broad spectrum (resistance to both inorganic and organic mercury compounds). The key enzyme in both phenomena is MerA (EC 1.16.1.1), the mercuric ion reductase, which reduces reactive, ionic mercury, Hg(II), to the less reactive, volatile metallic mercury vapor, Hg(0). Bacteria with broad spectrum mercury resistance also have the organomercurial lyase (MerB) which breaks the carbon−mercury bond of a broad range of organomercurials with a rate acceleration of 10^6-10^7 (*1*), yielding Hg(II) [subsequently converted to Hg(0) by MerA] and the corresponding protonated hydrocarbon. In Gram-negative *Enterobacteriaceae*, the *merB* gene typically occurs in plasmid-carried *mer* operons immediately 3′ of the *merA* gene (*2*). In *Pseudomonas* species *merB* occurs in a variety of operon structures (*3*−*5*), usually associated with transposons on plasmids. In Gram-positive bacteria, *merB* is also associated with transposons but may be chromosomal in *Bacillus* (*6*, *7*), *Clostridium*, and *Streptomyces* (*8*, *9*) and on plasmids in *Staphylococcus* (*10*).

The most extensively characterized MerB is encoded by the large, conjugative IncM plasmid R831b (*18*), originally isolated from *Serratia marcescens*, a member of the *Enterobacteriaceae*. Initial kinetic analysis using a MerA-coupled assay with partially purified MerB showed K_m values between 7 and 200 μM at substrate concentrations of 0.2−100 μM (*11*). Later studies using MerA-independent assay methods with highly purified MerB yielded K_m values between 0.5 and 3 mM and k_{cat} values between 0.7 and 240 min^{-1}, with weaker activity on alkyl mercurials than on aryl mercurials (*12*). Purified MerB was most active at pH 10.2 and required a greater than 2-fold excess of cysteine over substrate for sustained activity. MerB preferred cysteine to other thiols tested (thioglycolate, 2-mercaptoethanol, 2-aminoethanethiol, and dithiothreitol); glutathione was not examined (*11*, *12*).

On the basis of isotope effects and conservation of stereochemistry, Begley et al. (*13*) concluded that MerB acts by electrophilic substitution (S_E2), an unusual concerted mechanism that has only been proposed for two other enzymes: tryptophan indole-lyase (*14*) and orotidine 5′-monophosphate decarboxylase (*15*). Evidence for an S_E2

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[1] Abbreviations: CSH, reduced cysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; GSH, reduced glutathione; Hg, mercury; Hg(II), divalent mercury; IAM, iodoacetamide; IMAC, immobilized metal affinity chromatography; IncM, incompatibility group M (plasmid taxonomy classification); LMCT, ligand-to-metal charge transfer; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate; TCA, trichloroacetic acid.

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mechanism has previously been demonstrated for the degradation of organomercurials by nonenzymatic processes (*16*, *17*). In an S_E2 protonolysis, the mercurated carbon would gain a partial negative character as the Hg gains a partial positive character. Then a proton from the solvent or the protein would attack the carbon while the Hg attaches to an available negatively charged ligand such as a solvent halide or a solvent or protein thiolate.

To define the protein and solvent components of MerB-mediated protonolysis of PHMB, we have focused on the roles of the four cysteine residues of R831b MerB, each of which is essential for the phenylmercury resistance phenotype in vivo (*1*), and have assessed the kinetic activity, substrate/product binding, and turnover abilities of wild-type and mutant enzymes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. Plasmid pCT12 contains the OMR locus from R831 (*18*) cloned as an *Eco*RV fragment into the ampicillin-resistant, multicopy vector pBR322 (*19*). Plasmid pQZB1 was constructed by PCR amplification of *merB* from purified R831b (*18*) DNA. The MerB reading frame was fused at its C-terminus to a short linker (Leu-Glu) and a six-histidine tag (MerB-6His) and is expressed under control of the T7 promoter in the vector pET21b (Novagen Inc., Madison, WI). All strains were cultured on Luria−Bertani (L-B) broth at 37 °C, supplemented with ampicillin (100 μg/mL) or phenylmercuric acetate (25 μM) as needed.

Construction of Site-Directed Mutants. Site-directed mutants of the *merB* gene in pQZ1B were constructed as previously described (*20*). Each cysteine residue was changed to serine using the following sets of primers: C96S-UP, 5′-TGTATGCCTGGTCCGCGCTGGACAC; C96S-LOW, 5′-GTGTCCAGCGCGGACCAGGCATACA; C117S-UP, 5′-GTCTCATCGCATTCCGCTGCAACCG; C117S-LOW, 5′-CGGTTGCAGCGGAATGCGATGAGAC; C159S-UP, 5′-GTCAGTCCTTCTCTTGCCATGTACA; C159S-LOW, 5′-TGTACATGGCAAGAGAAGGACTGAC; C160S-UP, 5′-CAGTCCTTCTGTTCCCATGTACATT; C160S-LOW, 5′-AATGTACATGGGAACAGAAGGACTG. The underlined regions indicate the mutated codon. The primers B_*Nde*I (5′-TCACTACACATATGAAGCTCGCCCCATATATT) and B_*Xho*I (5′-TTTACTCGAGCGGTGTCCTAGATGACATG) were used in PCR reactions with each lower and upper primer, respectively, to generate the eight initial amplicands and also to fuse the amplicands by extension overlap. A near-zero background cloning method was used to generate the clones (*21*) that were sequenced, and strains carrying plasmids with the correct insertions were stored at −70 °C.

Protein Purification. BL21(DE3) strains containing pQZB1, pKPC96S, pKPC117S, pKPC159S, or pKPC160S were grown overnight (∼16 h) in Luria−Bertani broth at 37 °C with aeration to allow leaky gene expression. A liter of cell culture was harvested in a Beckman JA-10 rotor at 5000*g* for 10 min, and pellets were suspended in 30−40 mL of cold 20 mM sodium phosphate buffer, pH 7.5, with 500 mM NaCl, 10 mM imidazole, and 2 mM 2-mercaptoethanol. The suspended cells were lysed using two passes through a French press at 16000 psi and 4 °C. Lysates were cleared by centrifugation at 15000*g* for 30 min at 4 °C, and the supernatant was passed through a 0.2 or 0.44 μm filter. Approximately 50% of expressed protein was in the soluble fraction, except for C117S MerB, which was produced exclusively in inclusion bodies and was not examined further. Protein eluted from a $NiSO_4$-charged HiTrap chelating column (Amersham-Pharmacia, Uppsala, Sweden) at approximately 75 mM imidazole. The yield of pure MerB-6His after a typical IMAC purification was 120 mg from 1 L of culture, nearly 16% of total cell protein. To remove the ≤5% extraneous protein, samples were loaded on a Q10 column (Bio-Rad, Hercules, CA) containing Q-Sepharose, and protein was eluted using a gradient of 0−0.25 M NaCl over 50 mL. MerB-containing fractions after each purification step were pooled and exchanged by gel filtration into 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM DTT using a HiPrep 26/10 desalting column (Amersham-Pharmacia). Protein concentrations during purification steps were determined by Coomassie Plus Protein Assay reagent with BSA as a standard (Pierce, Rockford, IL) or by OD_{280} methods using a calculated $\epsilon_{280} = 23950\ M^{-1}\ cm^{-1}$ (*22*). These techniques agreed to within 5%. Molecular weights of purified proteins were determined by ESI-MS using a Sciax AP1+ (Perkin-Elmer, Shelton, CT) with prior HPLC fractionation.

Antibody Production. For Western detection we produced polyclonal antisera in rabbits to IMAC-purifed MerB-6His. Cells were removed by centrifugation, and serum was stored at −70 °C in small aliquots.

Cell Fractionation and Detection and Quantification of Proteins. Cell cultures in L-B broth were amended at mid-exponential growth phase ($OD_{600} = 0.6$) with 0.1 mM IPTG to induce chromosomal β-galactosidase and MerB-6His carried by pQZB1. Simultaneously, cultures containing R831b or pDU202/pCT12 were amended with 2 μM $HgCl_2$ every 30 min for 90 min to induce *mer* operon expression. Cell partitioning was achieved by osmotic shock (*23*) using 5 mM $MgSO_4$ rather than water in the final step. Cell fractions were electrophoresed on reducing SDS−PAGE gels using amounts of each fraction optimized for visualization of proteins of interest (1−5 μL of whole cells or cytoplasm/membrane; 15 μL of periplasm). After electrotransfer of proteins to PVDF membranes, Western analysis was performed with 1:2000 anti-β-lactamase (Eppendorf 5 Prime, Boulder, CO), 1:750 anti-β-galactosidase (Rockland Immunochemicals Inc., Gilbertsville, PA), and 1:1000 anti-MerB. Fluorescein-conjugated secondary antibody was used at 1:1000. Dried blots were scanned using a Fluorimager 575 (Molecular Dynamics Inc., Sunnyvale, CA), and densitometric measurements were performed using ImageQuant (version 1.2; Molecular Dynamics Inc.) using the spot finder feature for band identification and histogram peak for background correction (User's Manual, Molecular Dynamics Inc.).

Spectrophotometric Enzyme Assay. A facile spectrophotometric assay for MerB was developed on the basis of the Hg−cysteine ligand-to-metal charge transfer chromophore in the near-UV (*24*). The UV spectra of Hg with dithiol ligands peaks at 200 with a shoulder through 220 nm, but when Hg takes a third thiol ligand, the UV absorbance broadens, and Gaussian decomposition identifies a peak at 239 nm and a smaller, broader peak at 265 nm (*29*) with a very long, relatively flat tail extending to higher wavelengths.

To avoid interference from the aromatic substrate itself which absorbs strongly in the ca. 240 nm region, we used 300 nm where the extinction coefficient of the PHMB−thiol adduct was less than 3% of that for the Hg−thiol adduct with either cysteine or glutathione. The Hg−thiol extinction coefficients at 300 nm with either cysteine or glutathione increased linearly to ca. 20 mM thiol, consistent with an increasing proportion of trigonally coordinated species (*24*, *29*), and had pH maxima of ca. 8.5 (data not shown). For all conditions tested, standard curves at 300 nm using $HgCl_2$ obeyed Beer's law with R^2 values >0.99. For kinetic experiments, assay buffer (0.5 mL) containing 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5−20 mM cysteine, and between 0.1 and 1 mM PHMB was added to duplicate cuvettes and equilibrated in the dark at 37 °C for 10 min. The reaction was initiated in one set of cuvettes by addition of enzyme (0.5−10 μg), and the other set was used as a reference. The increase in absorbance at 300 nm was followed in a Cary 100 Bio spectrophotometer (Varian, Palo Alto, CA). Initial rates were approximated using the best-fit line to the time interval between 0 and 4 min or occasionally at the time interval when the slope was maximal, typically before 10 min. These rates were converted to specific activity on the basis of the concentration of protein used and were plotted on Origin Software (MicroCal, Northampton, MA) for double-reciprocal linear analysis and nonlinear fitting of the kinetic data. Protein samples were reduced with DTT and desalted by FPLC or spin columns into 100 mM Tris-HCl (pH 8.0) and 1 mM EDTA prior to all experiments. Routine DTNB analysis of such protein preparations immediately after gel filtration revealed each to be fully reduced and to have no detectable exogenous, nonprotein thiols. To test the effects of DTT on enzyme activity, assays were performed as above, using 1 mM PHMB and 20 mM cysteine or glutathione. DTT was added at varying concentrations prior to initiation by wild-type or C160S MerB at 1 or 3.8 μg, respectively.

HPLC Enzyme Assay. To corroborate and extend the spectrophotometric assay, we used HPLC to detect benzoic acid produced by protonolysis of PHMB. Using a solvent phase with 40% methanol and 0.1% acetic acid on a C18 reverse-phase column (Ultrasphere ODS, 4.6 × 150 mm; Beckman, Berkeley, CA) at 0.8 mL/min flow rate, the retention time of benzoic acid is about 11 min. The substrate (0.1−1 mM final) in 1.5 mL of assay buffer was equilibrated at 37 °C for 10 min in the dark and the assay begun by addition of freshly reduced and desalted enzyme. At various time points before and after enzyme addition 200 μL aliquots of the reaction were removed into an Eppendorf tube containing 20 μL of 100% TCA (to precipitate protein and stop reaction). Samples were filtered through 0.2 μm acetate or PTFE syringe filters (4 mm) into small-volume amber vials and then injected by an autosampler (Alcott 728, Norcross, GA). Enzyme-free controls were done in parallel to correct for TCA-mediated hydrolysis of PHMB under the assay conditions. The maximum observed rate was ca. 0.015 μM/min for 1 mM PHMB samples and at no time was the background hydrolysis greater than 15% of the active enzyme rate at the corresponding time point. Peak areas were generated automatically using an integrator (Spectra-Physics, San Jose, CA). Using absorbance at 230 nm for detection, as little as 0.2 nmol of benzoate in an injection volume of 100 μL was reliably measured, and the peak area was linear between 0.2 and 10 nmol of benzoate. Kinetic parameters were determined as above.

Free Thiol Determination. Protein was reduced with 10 mM DTT for 20 min and desalted into 20 mM sodium phosphate buffer, pH 7.5, and 0.5 mM EDTA using a 5 mL HiTrap desalting column (Amersham-Pharmacia). The concentration of protein was determined by OD_{280}. Twenty-five microliters of 10 mM DTNB in 0.2 M sodium phosphate buffer, pH 7.5, was added to 500 μL of 10−20 μM protein, and the absorbance at 412 nm was recorded every 5 min and was stable for 30 min. Cysteine was used to determine an extinction coefficient of $\epsilon_{412} = 13560\ M^{-1}\ cm^{-1}$ for TNB, similar to the reported values of 13600−14140 $M^{-1}\ cm^{-1}$ (*25*).

Covalent Modification of MerB Thiols. Proteins were reduced and desalted, and concentrations were determined as above, except that 20 mM Tris-HCl (pH 8.0) was used. Fifty microliter samples were incubated (or not) with PHMB at 1:1, 2:1, and 4:1 [PHMB]:[protein] for 5 min at 24 °C. *N*-Ethylmaleimide (NEM) or iodoacetamide (IAM) was added to 10 mM (protein concentrations were <190 μM), and the modification proceeded for 20 min at 24 °C in the dark. Samples were immediately desalted by centrifuging through a spin column consisting of a 1 mL syringe containing Sephadex G-25 resin equilibrated with 100 mM ammonium bicarbonate. Then 1 μL of modified trypsin (0.1 mg mL^{-1}, Promega catalog no. V5111) was added to 9 μL of the column effluent protein solution, and this reaction was incubated overnight at 37 °C. Tryptic peptides were diluted 1:1 with saturated dihydroxybenzoate in 100 mM ammonium bicarbonate and spotted onto a sample stage containing crushed crystals of dihydroxybenzoate. After being dried, they were analyzed on a Bruker Reflex II MALDI-TOF mass spectrometer in reflectron mode. Peaks were compared to predicted patterns of tryptic digestion of MerB as determined by the program MS-digest (*26*).

RESULTS

Insights from the Organomercurial Lyase Family. GenBank presently records 27 sequences homologous to R831b MerB from over a dozen eubacterial species; none have homologues with any other protein with an identified function; i.e., MerB is a unique enzyme. Among these 27 sequences, those with >90% identity fell into 10 distinguishable groups, and a single representative of each group is included in Figure 1A. There is good sequence conservation only in the central region of MerB including three cysteines at positions 96, 117, and 159 (R831b MerB numbering). In the majority of the Gram-negative examples C159 is part of a vicinal cysteine pair with C160; in the Gram-positive MerB proteins the vicinal pair is at or very near the carboxy terminus.

Phylogenetically, there are three distinct subfamilies of MerB's (Figure 1B), and this grouping is also reflected in the secondary structure predictions (Figure 2). In secondary structure groups A and B, helices dominate the termini and sheets dominate the center of the primary sequence; two of the three conserved cysteines, Cys 117 and Cys159 (R831b numbering), are predicted to lie in regions of similar secondary structure. The most distantly related MerBs from

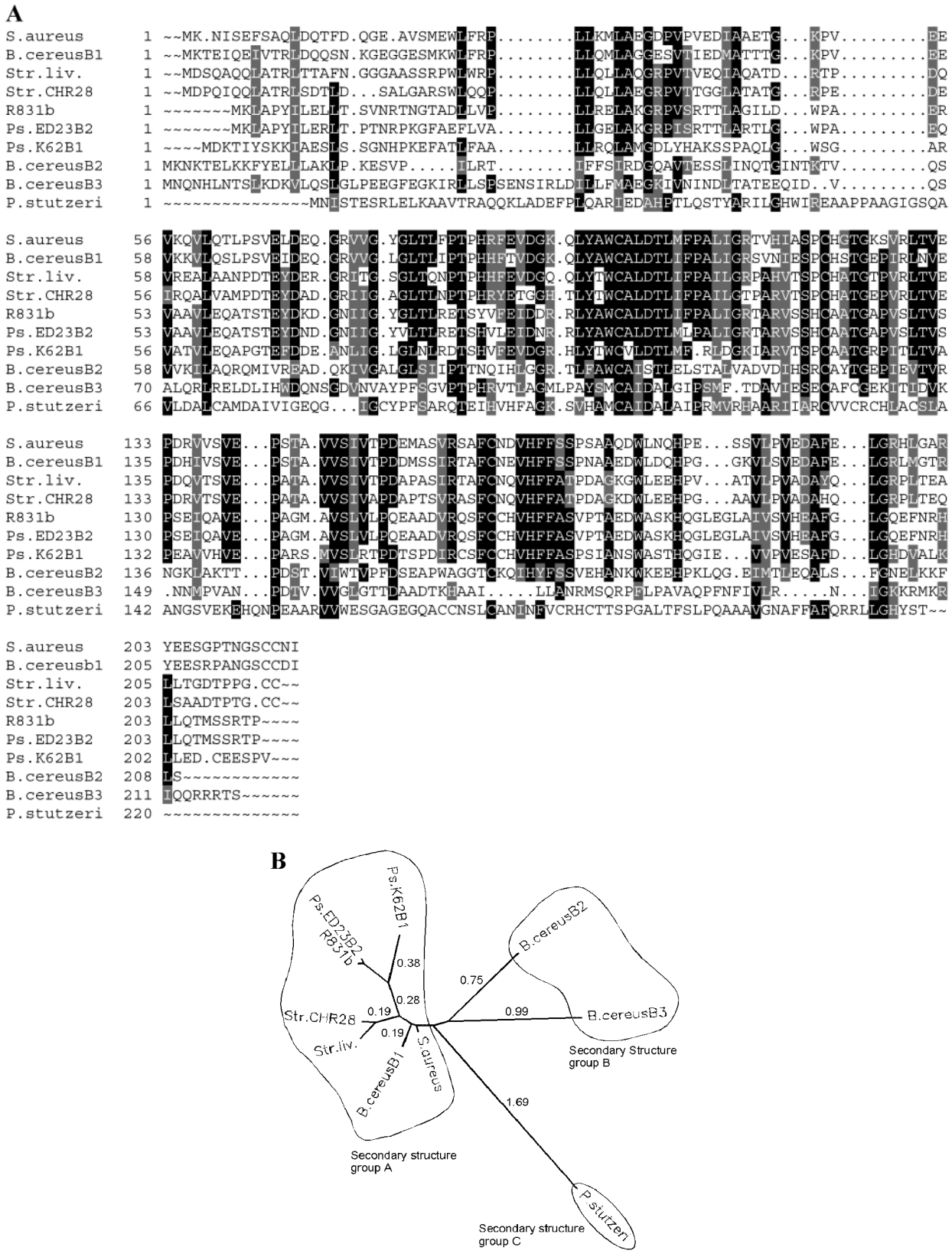


FIGURE 1: (A) Unique MerB sequences. Each listing is a representative of one or more other sequences (not shown) which are >90% identical to it. The listed sequences are <90% identical to each other. (B) Unrooted phylogenetic tree based on the neighbor-joining method (*41*). Branch lengths are indicated on longer branches. For predicted secondary structure groups, see Figure 2.

Bacillus cereus and *Pseudomonas stutzeri* have only been defined by phenotype but have not been characterized biochemically (*5*, *6*). The predicted secondary structure of the *P. stutzeri* MerB bears no resemblance to that of any of the others. The most populous phylogenetic cluster (left side of tree diagram, Figure 1B) includes Gram-negative and both high- and low-GC Gram-positive bacteria; this point will be considered further in the Discussion.

MerB Is a Cytosolic Enzyme. Although the primary sequence of MerB did not indicate that it was a secreted

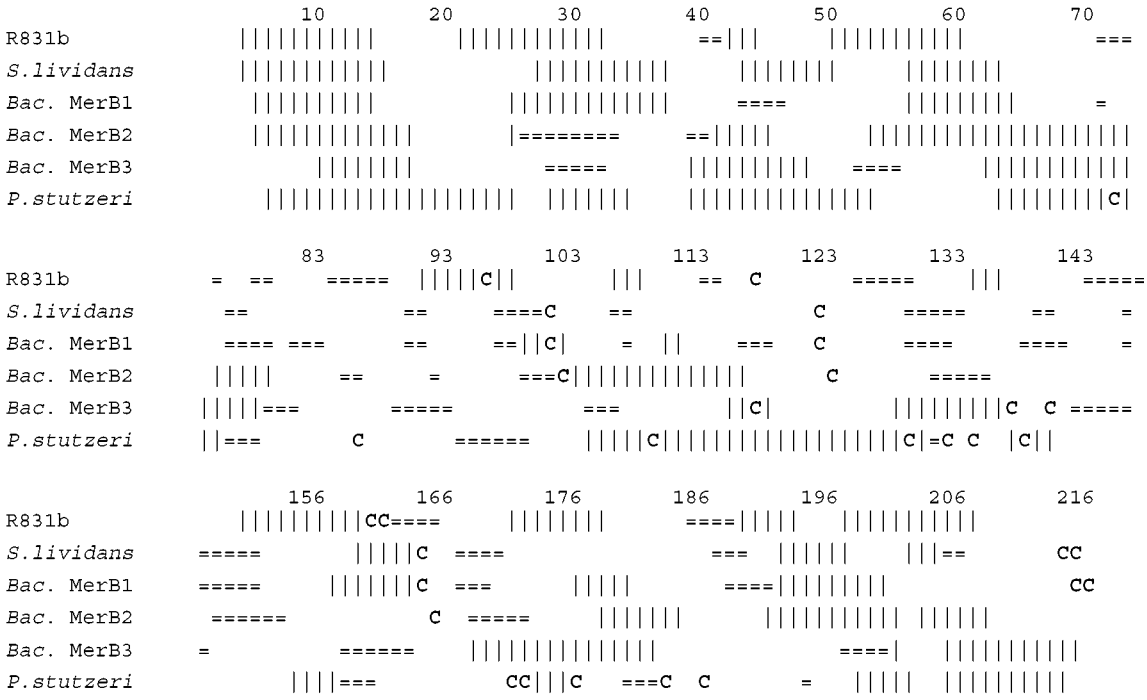


FIGURE 2: Secondary structure prediction for MerB proteins based on a consensus method (*27*). Key: =, β-sheet; |, α-helix; C, cysteine residue. Sequence positions in R831b MerB are above the top line. Group A: R831b, *S. lividans*, and *Bacillus* MerB1. Group B: *Bacillus* MerB2 and *Bacillus* MerB3. Group C: *P. stutzeri*. See phylogenetic tree, Figure 1B.

Table 1: MerB Is a Cytosolic Enzyme

protein	% protein[a] in periplasm	% activity[b] in periplasm	% protein[a] in cytoplasm	% activity[b] in cytoplasm
MerB[c]	3.5 ± 0.10	0	108 ± 32	124
MerB-6His	0.78 ± 0.18	0.43	99 ± 7	106
β-galactosidase	0.78 ± 0.12	nd[d]	93 ± 14	nd[d]
β-lactamase	59 ± 4.0	nd[d]	37 ± 12	nd[d]

[a] Signals were derived from densitometric intensity of Western bands and normalized to the signal from whole cells by dividing by the fraction volume added to the gel. Percentages are relative to whole cell signal (see Figure 1). [b] Enzyme activity of whole cells and of spheroplasts was determined using sonicated, cleared lysates. Data with standard deviations are the average of three experiments. [c] Values for wild-type MerB are the average of the three strains examined which produce this protein from plasmids R831b, pCT12, and pCT12(pDU202). [d] Not determined.

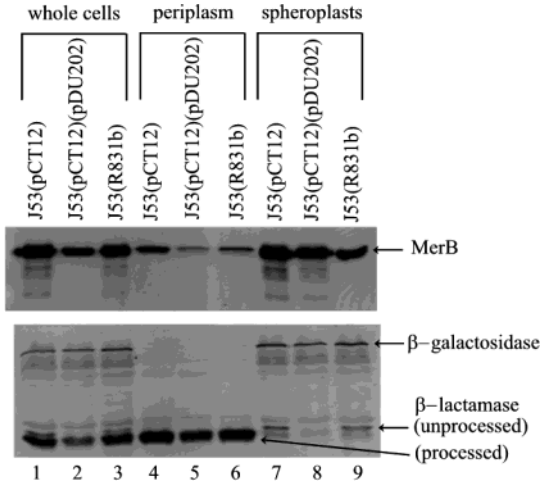


FIGURE 3: MerB partitioning in *E. coli* cells. Membranes were incubated with anti-MerB (upper panel) or anti-β-galactosidase and anti-β-lactamase (lower panel) polyclonal antibodies. Lanes: 1−3, whole cells; 4−6, periplasm; 7−9, spheroplasts. Lanes 1, 4, and 7 contain strain J53(pCT12), lanes 2, 5, and 8 contain strain J53-(pCT12)(pDU202), and lanes 3, 6, and 9 contain strain J53(R831b). Periplasm was loaded at 3-fold (lower panel) or 10-fold (upper panel) the volume of the other fractions. Markers: MerB, 22.4 kDa; β-galactosidase, 116 kDa; unprocessed β-lactamase, 22 kDa; processed β-lactamase, 20.6 kDa.

protein, its cellular location had never been determined. In strains carrying the original monocopy plasmid R831b, a multicopy plasmid, pCT12, or both multicopy pCT12 and a monocopy plasmid with a narrow spectrum resistance locus (pDU202), the periplasmic fraction had an average of 3.5% of the total cellular MerB (Table 1 and Figure 3A, lanes 4−6). The cytosolic marker enzyme, β-galactosidase, was well retained (93%) in the spheroplasts (=, the cytosol), with nearly undetectable bands in the periplasmic fractions (Figure 3B, lanes 1−6), indicating that the osmotic shock procedure used did not disrupt the cell wall (i.e., the murein and the cytoplasmic membrane). In contrast, at least 59% of the processed β-lactamase (a periplasmic marker enzyme) was in the periplasm, and only unprocessed β-lactamase remained associated with shocked spheroplasts (Table 1 and Figure 3B, lanes 4−9), indicating that our shock procedure effectively released bona fide periplasmic proteins. Moreover, no unprocessed β-lactamase was detectable in periplasm, providing further evidence that the shock procedure did not disrupt the cell wall.

Periplasmic preparations from the His-tagged overexpressor strain BL21(pQZB1) contained only 0.78% of the whole cell MerB, similar to the amount of β-galactosidase in the periplasm (Table 1). No significant difference was found between the periplasmic concentration of MerB in strains containing *merB* alone and in strains that also contain the entire *mer* operon (compare lanes 4 and 6, Figure 3), indicating that none of the other proteins from either narrow

Table 2: Effects of Cysteine or Glutathione on Wild-Type MerB Activity Using a Spectrophotometric Assay with PHMB[a]

	cysteine			glutathione		
[thiol] (mM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)
5	0.31	3.8	12.4	0.11	1.1	10.1
10	0.40	5.0	12.2	0.20	2.4	11.7
20	0.60	5.4	8.9	0.37	4.5	12.2

[a] In addition to the indicated thiol, assays contained 100 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.1−1 mM PHMB. Data are the average of two experiments (standard deviations less than 10%). K_M and k_{cat} values were determined by nonlinear fitting of averaged data to a hyperbolic Michaelis−Menten equation.

Table 3: Kinetic Parameters of Wild-Type and Mutant MerB Proteins Using the Spectrophotometric and HPLC Assays with PHMB as a Substrate[a]

	spectrophotometric assay[c]			HPLC assay[d]		
protein, thiol[b]	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\,s^{-1}$)
WT, CSH	0.60	5.4	8.9	0.73	5.9	8.0
WT, GSH	0.37	4.5	12.2	0.46	4.8	10.4
C96S, CSH	na	na	na	nd	nd	nd
C159S, CSH	na	na	na	nd	nd	nd
C160S, CSH	0.43	2.2	5.0	0.20	2.0	9.9
C160S, GSH	0.14	0.65	4.7	0.16	0.6	3.4

[a] In addition to the indicated thiol at 20 mM assays contained 100 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.1−1 mM PHMB. Data are the average of two experiments (standard deviations less than 10%). K_M and k_{cat} values were determined by nonlinear fitting to a hyperbolic Michaelis−Menten equation. [b] CSH, cysteine; GSH, glutathione. [c] na, no detectable activity. [d] nd, not done.

or broad spectrum operons influences partitioning of MerB. MerB in the cytoplasm of R831b was nearly 1% of total cell protein (data not shown), indicating that it is highly expressed even from this low copy plasmid.

Effects of Physiological Thiols, Cysteine and Glutatione, on MerB. MerB's cytosolic location is consistent with its previously noted dependence in vitro on millimolar buffer thiol (*12*, *13*). Since only one of the previously tested thiols (cysteine) is a physiological thiol and since glutathione, not cysteine, is the thiol buffer for *Escherichia coli* and other *Enterobacteriaceae*, we examined MerB activity in cysteine (normally present in *E. coli* at submicromolar levels) and glutathione (normally present at 5 mM in *E. coli*) (*33*) (Table 2). For both thiols MerB's activity on PHMB increased with increasing thiol concentration up to 20 mM (Table 2) and plateaued or declined at higher concentrations up to 50 mM (data not shown). The K_M values for the glutathionyl derivatives of PHMB are modestly lower than the cysteinyl-PHMB derivatives. However, with cysteine MerB had slightly higher turnover rates than with glutathione. The greatest differences between the two thiols for both K_M and k_{cat} occurred at 5 mM thiol, the typical physiological range for glutathione in Gram-negative bacteria and for cysteine in Gram-positive bacteria. The catalytic efficiencies (k_{cat}/K_M) afforded by each thiol are nearly equivalent, diverging only slightly at very high (nonphysiological) thiol concentrations.

The spectrophotometric assay is facile, but the extinction coefficient of the mercury−thiol LMCT increased with thiol concentration (see Experimental Procedures), so we also used HPLC as an independent assay to detect benzoate production from PHMB. For wild-type MerB in the HPLC assay the K_M values for PHMB using either thiol differed by only 20%, and the k_{cat} values differed from the spectrophotometric assay by less than 10% (Table 3). Thus, in vitro R831b MerB functioned equally well in protonolysis of PHMB with either of the two common physiological thiols, cysteine or glutathione. This is in notable distinction to the effect on MerB of the nonphysiological thiol, DTT (see below). Since the highest activity for each thiol was obtained at 20 mM thiol, we used this routinely.

Kinetic Parameters of MerB Cysteine Mutants. The cysteines of MerB are functionally distinct. Serine replacements at highly conserved Cys96 and Cys159 resulted in proteins that had no detectable activity with cysteine (Table 3) or with glutathione (data not shown). In contrast, serine replacement at nonconserved Cys160 resulted in an enzyme which retained some enzymatic activity and that activity showed a slight thiol preference (Table 3). The K_M values for PHMB in both cysteine and glutathione for C160S were consistently lower compared to the wild type with the respective thiol. Like wild type, the K_M for PHMB−glutathione of C160S was always lower than for PHMB−cysteine and for the mutant protein even approached the K_M values observed in the MerA-dependent assay (*11*). However, unlike the wild type, the difference in k_{cat} for the two thiols was much greater. In both assay modes, C160S retained ca. 37% of wild-type activity in cysteine but only ca. 13% of wild-type activity when glutathione was the buffer thiol. Nonetheless, the modest decrease in K_M in C160S compensated for the lower turnover rate, yielding an overall catalytic efficiency for the mutant protein similar to the wild type with cysteine and impaired by only 70% in glutathione. Cys160 appears to have a distinct and less critical role in MerB's mechanism than either Cys159 or Cys96.

Inhibition by DTT. In our early work we stored purified MerB in 10 mM DTT until we discovered that DTT carryover from the storage buffer was the cause of a 3−10 min lag in the initial phase of the protonolysis reaction with cysteine in the assay buffer (data not shown). Previously, DTT was shown to be least effective in stimulating MerB activity of several thiols tested (*11*) but was not actually demonstrated to inhibit MerB. We found that inhibition of PHMB protonolysis by DTT differed when cysteine or glutathione was in the assay buffer (Figure 4). The concentration of DTT at which MerB activity on PHMB is halved (IC_{50}) was 3.4 μM with 20 mM glutathione and 19 μM with 20 mM cysteine. In 20 mM cysteine the mutant C160S exhibited an intermediate IC_{50} of 8.4 μM DTT. In all cases, the protonolysis rate at maximum DTT inhibition was about 10% of the uninhibited rate. Even with DTT as the only thiol in the assay, the rate did not go to zero, although the k_{cat} value of MerB in 10 mM DTT was just 2% that in 10 mM cysteine and the K_M value comparable to cysteine (data not shown). Note that these mixed thiol assays were done with the spectrophotometric method and reaction rates derived using the extinction coefficient of the most abundant thiol (either glutathione or cysteine) which was present at a minimum 100-fold molar excess over DTT. Although the ϵ_{300} for Hg−DTT is 2-fold greater than those of Hg−glutathione or Hg−cysteine, under these conditions absorbance of Hg−DTT would contribute only a small error in the specific activities measured. The possible nature of the

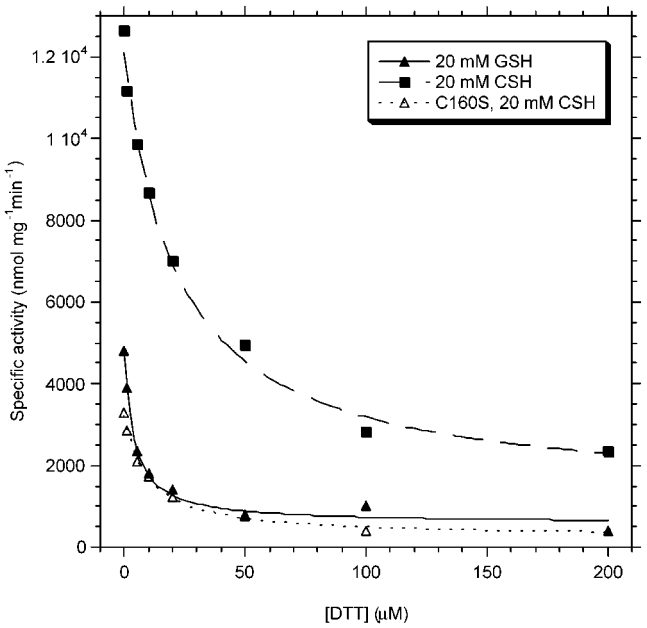


FIGURE 4: Inhibition of MerB by DTT. Proteins were reduced in 10 mM DTT, and then DTT was rapidly removed by desalting in a spin column (see Experimental Procedures). The protein was immediately assayed spectrophotometrically with 1 mM PHMB as the substrate in buffers containing the indicated thiols. The inhibitor DTT was added at the indicated concentrations just prior to enzyme addition. Symbols: (■) wild-type MerB in 20 mM glutathione; (▲) wild-type MerB in 20 mM cysteine; (△) C160S MerB in 20 mM cysteine.

Table 4: Thiols Available to DTNB in Wild-Type and Mutant MerB Proteins[a]

	thiols/monomer			
	WT	C96S	C159S	C160S
no addition	3.11 ± 0.15	2.045 ± 0.21	2.21 ± 0.02	1.63 ± 0.07
guanidine	3.90 ± 0.01	3.445 ± 0.01	3.06 ± 0.03	2.55 ± 0.00

[a] Protein concentration determined by OD_{280}. Data with standard deviations are the average of two or more experiments.

DTT-inhibited enzyme complex will be discussed below.

Protein Thiol Availability to Reaction with DTNB. Reduced and desalted wild-type MerB reacted with three molecules of DTNB whereas similarly prepared C96S, C159S, and C160S MerB reacted with two molecules of DTNB (Table 4), indicating that one thiol of MerB was unreactive or sterically inaccessible to DTNB. We infer that the DTNB-unreactive residue is Cys117. When MerB was denatured in guanidine, DTNB reacted with four thiols in wild type, and an average of three in the mutants, indicating that lack of reactivity in Cys117 arises from its being buried rather than liable to irreversible oxidation.

Covalent Thiol Modification. Inactivation of MerB by the covalent thiol-modifying agents iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) occurred in a time-dependent fashion, decreasing enzymatic activity by 85−90% within 10 min using the spectrophotometric assay (data not shown), in contrast to previous reports where MerB was not inactivated by iodoacetate (*12*). MALDI-MS analysis of tryptic digests of IAM-treated proteins showed that all MerB cysteines were modified by IAM (Table 5). However, the degree of modification of Cys117 was less than the other Cys-containing peptides; Cys117 fragments were always visible as a mixture of modified and unmodified peptides, whereas for other cysteine-containing fragments, the unmodified peptides were frequently undetectable after IAM was added (data not shown). In the case of C159S MerB, Cys117 was not modified by IAM (Table 5), suggesting that Cys117 is less available for reaction in the C159S protein, although not so in the C160S mutant. NEM reacted with all of the cysteine residues, but some unmodified peptides were always detectable. No disulfide-linked tryptic fragments were observed either in the free protein or in the PHMB derivatives described below.

Protection by the Substrate from Covalent Thiol Modification. Although MALDI-MS analysis is not quantitative, it has been used to draw conclusions from sizable and consistent differences in peptide modification levels (*34*, *35*). MALDI-MS analysis of peptides resulting from IAM or NEM treatment of reduced and desalted proteins generally resulted in clear covalent modification of all cysteines in wild-type and mutant proteins (Table 5). The Cys159/Cys160 peptide was always seen as doubly modified by IAM or NEM (unless its visibility was precluded by overlap with another peptide; see footnote to Table 5). Only in the C159S mutant was Cys117 not modified by IAM and Cys160 only weakly modified by NEM.

Equimolar PHMB did not preclude covalent modification of any cysteine reactive in its absence, and most peptides of the mutant proteins were seen as mixtures of PHMB-modified and IAM- or NEM-modified fragments, indicating that all were accessible to the substrate. We did not see 2 × PHMB or mixed PHMB + IAM or PHMB + NEM adducts of the Cys159/Cys160 peptide. A 2-fold molar excess of PHMB prevented covalent modification of the Cys159/Cys160 peptide in the wild type and modification of that peptide and the Cys96 peptide in the C160S mutant. Otherwise, Cys96 and, notably, Cys117 remained accessible to covalent modification in all other cases even at a 2-fold molar excess of PHMB.

The patterns of PHMB adducts also revealed distinctions among the cysteines. In the wild type the Cys159/Cys160 always had an Hg adduct, never a PHMB adduct, suggesting that the reduced wild-type protein without exogenous thiols can effect protonolysis but cannot release the Hg(II) product from this peptide. None of the MerB mutants (even the partially active C160S mutant) displayed such Hg adducts when presented with PHMB (Table 5) but all readily formed Hg adducts (predominantly on the Cys159/Cys160 peptide) when presented with 1:1 or 2:1 $HgCl_2$ (data not shown), indicating that the PHMB-derived Hg adducts at C159/C160 in the wild type were not caused by Hg(II) contamination in the PHMB stock. In contrast to the wild type, only PHMB adducts occurred at 1:1 PHMB in C96S (at Cys117) and especially in C159S where all remaining cysteines formed PHMB adducts at both 1:1 and 2:1 PHMB, consistent with the absence of any protonolysis activity in these mutants.

These observations did not likely result from reassociation of PHMB during trypsinolysis because the digest buffer did not contain thiols, and under such conditions dissociation of PHMB from a protein cysteinyl occurs rarely. These observations suggest that the PHMB substrate can associate stably with any of MerB's cysteines including Cys117. In contrast, Hg(II) associates most stably with the intact vicinal cysteine pair at 159/160, although we cannot conclude that

Table 5: Protection of Cysteine Residues from Covalent Modification by Substrate PHMB[a]

	wild type						C96S mutant			
	C96		C117		C159/C160		C117		C159/C160	
	NEM	IAM	NEM	IAM	NEM	IAM[b]	NEM	IAM	NEM	IAM[b]
no addn	■	■	■	■	■	np	■	■	■	np
1:1 PHMB	■	■	■	■	■[c]	np[c]	■	■[d]	−	np
2:1 PHMB	−	■	−[d]	■	×[c]	np[c]	■[d]	■[d]	■[d]	np

	C159S mutant						C160S mutant					
	C96		C117		C160		C96		C117		C159	
	NEM	IAM	NEM	IAM	NEM[b]	IAM	NEM	IAM	NEM	IAM	NEM	IAM
no addn	■	■	■	×	np	■	■	■	■	■	■	■
1:1 PHMB	■[d]	■[d]	■[d]	×[d]	np[d]	■[d]	−	■	−	■	■	■
2:1 PHMB	■[d]	−	■[d]	−	np[d]	−	−[d]	×	−[d]	■	×[d]	×

[a] Reduced and desalted protein samples were incubated with various ratios of PHMB:protein before 10 mM *N*-ethylmaleimide or iodoacetamide was added. Reacted proteins were desalted and digested overnight with modified trypsin (Promega), and peptides were detected by MALDI-MS. Unmodified wild-type peptide sizes: C96, 2036 Da; C117, 4250 Da; C159/C160, 2498 Da. Genotypes: wild type, C96S, C159S, and C160S. Peptides: C96, C117, C159, and C160. Reagents: NEM and IAM. Symbols: ■, peptide covalently modified; ×, peptide not covalently modified; −, results not consistent between two experiments. [b] np, detection of covalent adduct not possible because it overlaps with a strong peak at 2610 Da arising from the tryptic peptide of residues 178−201. [c] Hg adduct (200 Da) present on the unmodified peptide. [d] PHMB adduct (320 Da) present on the unmodified peptide.

Table 6: Release of Benzoate from PHMB and from PHMB−Thiol Complexes by Wild-Type and Mutant MerB Proteins[a]

	mol of benzoate produced/mol of protein[b]			
substrate	wild type	C96S	C159S	C160S
PHMB, 150 μM	0.979 ± 0.033	0.007 ± 0.004	0.005 ± 0.010	0.934 ± 0.002
PHMB, 500 μM	0.714 ± 0.003	nd	nd	0.846 ± 0.031
PHMB−cysteine, 500 μM	2.496 ± 0.004	nd	nd	1.847 ± 0.041
PHMB−glutathione, 500 μM	1.447 ± 0.025	nd	nd	1.092 ± 0.006

[a] Proteins were incubated with the PHMB or complexes for 10 min at 24 °C in the dark. The reaction was stopped, and protein was precipitated by adding trichloroacetic acid to 10%. The reaction mixture was centrifuged and filtered (0.2 μm) prior to quantitation of benzoate by HPLC. Protein concentrations were between 50 and 72 μM. Values are the average of two experiments. [b] nd, not done.

it originates there since it might migrate into this stable dithiol coordination through interpeptide exchange even in the absence of buffer thiols.

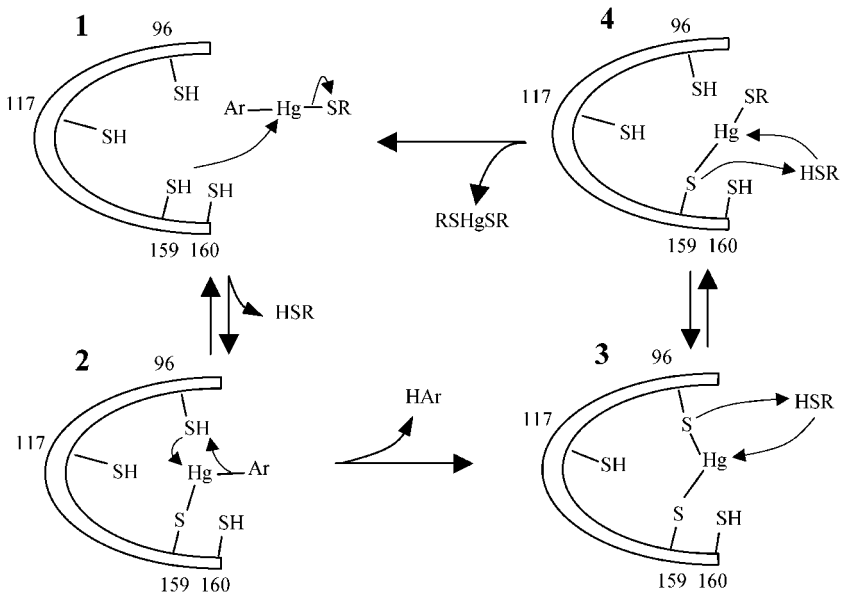
Single Turnover of PHMB and PHMB−Thiol Adducts. We used HPLC to quantify benzoate released from PHMB in order to test the possibility that reduced MerB can complete a single protonolysis in the absence of exogenous thiols. We exposed freshly reduced and desalted enzymes to varying amounts of PHMB or to PHMB−cysteine or PHMB−glutathione complexes. Reduced wild-type MerB generated 0.98 mol of benzoate/mol of protein when incubated with a 3-fold excess of PHMB (Table 6). The turnover dropped to 0.71 at a 10-fold molar excess of PHMB, possibly due to inhibition by excess PHMB. C160S showed a similar turnover capacity of 0.93 and 0.85 mol of benzoate/mol of protein at 3- and 10-fold excesses of PHMB, respectively. Even at higher excess PHMB no additional benzoate product was produced between 30 s and 10 min (data not shown). Mutants C96S and C159S MerB did not generate benzoate from PHMB above background levels. When wild-type MerB reacted with an equimolar PHMB−cysteine complex at a 3-fold molar excess of substrate:protein, it produced 2.50 mol of benzoate/mol of protein, and C160S MerB produced 1.85 mol of benzoate/mol of protein. Using an equimolar PHMB−glutathione complex, the efficiency of turnover for both wild-type and C160S enzymes was ca. 41% lower than with PHMB−cysteine.

DISCUSSION

Phylogenetics of MerB. At present, MerB seems to be a unique protein since its primary sequence is not significantly similar to anything else in currently available databases. However, several subtypes of extant MerB proteins are discernible by standard phylogenetic methods. These phylogenetic subtypes also differ in their predicted secondary structure, leaving open the possibility that an organomercurial lyase activity has arisen more than once among bacteria. The most closely related members of the present MerB family have three conserved cysteines, and all have one or more additional cysteines. As cysteines are the most likely protein ligands for Hg, we focused on the role(s) of the cysteines in our work.

Cellular Partitioning of MerB and Its Implications for the Roles of MerB Cysteines. Gram-negative eubacteria have physically and biochemically distinct compartments: the interior, highly reducing cytoplasm and the more exterior, oxidized periplasmic space which lies between the inner and outer membranes of bacteria of this type. When we began this work, MerB was the only protein of the mercury resistance operon whose cellular location had not been determined. Although its lack of an obvious signal sequence indicated that MerB was very likely a cytosolic protein, it was important to establish this point unequivocally before beginning biochemical studies of the protein in order to anticipate the redox state of the cysteines in the native protein. Our clear finding that MerB is cytosolic is consistent with our observation that all four cysteines are present as thiols in the denatured protein. And, since only three thiols are available for modification in the native protein, one of them must be buried or otherwise altered in its reactivity by the protein fold. On the basis of genetic and biochemical data we concluded that, despite its being highly conserved and, therefore, an obvious metal ligand, Cys117 is relatively nonreactive compared to the other cysteines of R831b MerB.

Scheme 1



MerB Uses Either Common Physiological Thiol. The similar values of k_{cat}/K_M using glutathione and cysteine suggest that R831b MerB is effective with *E. coli*'s natural intracellular reducing agent, the tripeptide glutathione, as well as with the smaller cysteine. The 2−3-fold lower K_M value using glutathione indicates that it is preferred over cysteine, which makes sense as there is little free cysteine available in *E. coli* (*33*). Since MerB protonolyzes PHMB as a cysteine or glutathione complex, its active site is not severely constrained with respect to substrate size, in agreement with the earlier finding of similar K_M values for a wide range of aryl and alkyl mercurials as cysteine complexes (*12*, *13*). The distinct phylogenetic groups of MerB may reflect evolutionary adaptations to the cytosolic reducing agents used by the host: *Bacillus subtilis* and *Staphylococcus aureus* use cysteine, *E. coli* and *Pseudomonas aeruginosa* use glutathione, and *Streptomyces* uses mycothiol (*33*). MerB inhibition by the widely used dithiol reductant DTT was surprising. It may be that, unlike the physiological monothiols, DTT is not readily able to remove the Hg(II) product from the protein. Biochemical work with other dithiols will test this hypothesis, and the NMR structure of the DTT−Hg−MerB complex currently in progress will elucidate this point.

Model for the MerB Reaction Mechanism. The phylogeny of MerB and our biochemical observations suggest some refinements to the S_E2 mechanism proposed by Begley et al. (*12*, *13*), and these are summarized in Scheme 1. Of the three highly conserved cysteine residues, Cys96 and Cys159 are more accessible than Cys117, loss of Cys160 decreases the K_M for PHMB, the Cys159/Cys160 peptide provides ligand(s) for the product Hg(II), and in the absence of Cys159, the remaining cysteines more readily form adducts with PHMB. These observations suggest that Cys159 reacts more readily with PHMB than the other MerB cysteines. Thus, for simplicity, we propose that Cys159 provides a thiol ligand to both the substrate and the Hg(II) product; i.e., it is occupied by Hg throughout the reaction course. In step 1, Cys159 transfers its proton to and displaces the thiolate ligand (SR) from PHMB. Previous research has suggested that additional electronegative ligands to the Hg of the substrate activate the C−Hg bond to enhance protonolysis (*38*, *39*). This is a likely role of one or more of the other cysteines such as Cys96 which (step 2) may form a ligand to Hg(II) with its sulfur atom and concomitantly donate its proton to the hydrocarbon moiety (Ar) of PHMB. On the basis of sequence conservation, possible additional or alternative activating ligands and/or proton donors include Tyr93 or Cys117 residues. Although it is not uncommon for thiol groups to be either unreactive or slowly reactive with agents such as DTNB (*25*), Cys117's failure to react at all with DTNB, its weak reaction with the polar IAM, and strong reaction with the small, hydrophobic NEM are consistent with its being buried and having primarily a structural, rather than catalytic, role. A structural rather than catalytic role for Cys117 is also consistent with the insolubility of mutant proteins containing either serine or alanine at position 117.

Removal of the Hg(II) product takes two additional steps (steps 3 and 4) beyond protonolysis, each requiring a buffer thiol. In the absence of exogenous thiols and using nonthiolated substrate, the enzyme gets stuck at step 3 holding the Hg(II) with at least two of its cysteines. Note that we did not detect Cys96−Hg adducts by MALDI-MS after PHMB exposure of the wild-type protein, but this may be because formation of dicoordinate Cys159−Hg−Cys160 is energetically favored after trypsinolysis. When reduced MerB is presented with PHMB−thiol complexes, it can protonolyze two of them because the first Hg(II) product can be removed from the enzyme by the two thiols released from two PHMBs; the second Hg(II) remains bound to MerB, inhibiting further turnovers.

That the reduced C160S mutant protein readily turns over a single PHMB in the absence of exogenous thiols indicates that Cys160 is not the proton donor and is also not required for substrate binding. Indeed, its consistently lower K_M values compared to wild type suggest that its loss may improve substrate binding, especially in a glutathione buffer (Table 3). However, since C160S is partly deficient in multiple turnovers of PHMB−Cys and PHMB−GSH complexes (Table 6) and has a considerably diminshed k_{cat}, Cys160 may

facilitate release of the Hg(II) product to buffer thiols. In other *mer* operon proteins, such as MerA (*36*) and MerT (*37*), closely spaced cysteine pairs facilitate movement of Hg(II) from buffer thiols onto protein thiols. The vicinal cysteines of MerB might facilitate the opposite reaction: transfer of the Hg(II) product from the protein onto buffer thiols. However, because Cys160 is not highly conserved in MerB proteins, we did not give it any essential role in this present model. DTT may interfere at step 3 or 4, possibly by forming a stable trigonal intermediate with the Hg−Enz complex (*40*). The availability of all MerB cysteines for formation of PHMB adducts suggests a spacious and/or very plastic active site which might accommodate simultaneously the substrate and one or more buffer thiols.

In summary, we have found that the four thiols of the paradigmatic enterobacterial MerB have distinct roles in protonolysis of the model organomercurial, PHMB. The proton donor for this reaction is not the solvent but a MerB residue, possibly conserved Cys96. In the absence of buffer thiols the Hg(II) product is trapped by at least two MerB cysteines, most likely Cys159-Cys160. Conserved Cys117 probably plays a structural rather than catalytic role, and nonconserved Cys160 may impede substrate binding but facilitate product release. Structural studies currently in progress will provide a foundation for further biochemical analyses and genetic manipulation of this unusual, environmentally important enzyme.

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