A Mercuric Ion Uptake Role for the Integral Inner Membrane Protein, MerC, Involved in Bacterial Mercuric Ion Resistance*

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Lena Sahlman, Wendy Wong, and Justin Powlowski

From the Department of Biochemistry, Umeå University, S-901 87, Umeå, Sweden and the Department of Chemistry and Biochemistry, Concordia University, Montreal, Quebec H3G 1M8, Canada

Bacterial detoxification of mercuric ion depends on the presence of one or more integral membrane proteins (MerT and/or MerC) whose postulated function is in transport of Hg$^{2+}$ from a periplasmic Hg$^{2+}$-binding protein (MerP) to cytoplasmic mercuric reductase. In this study, MerC from the Tn21-encoded mer operon was overexpressed and studied in vesicles and in purified form to clarify the role played by this protein in mercuric ion resistance. MerC-containing vesicles were found to take up mercuric ion independently of MerP. Since uptake correlated with the level of MerC expression was unaffected by osmotic pressure, and was only partially decreased in the presence of 0.05% Triton X-100, the observed uptake appears to represent mainly binding to MerC. Binding was inhibited by thiol-specific reagents, consistent with an essential role for cysteine residues. The essential thiol groups were inaccessible to hydrophilic thiol reagents, whereas hydrophobic reagents completely abolished Hg$^{2+}$ binding. These observations are consistent with the predicted topology of the protein, wherein all 4 cysteine residues are either in the cytoplasm or the bilayer. A role for MerC in Hg$^{2+}$ transport is thus also likely. Based on these results, a modified model for bacterial Hg$^{2+}$ transport is proposed.

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**Experimental Procedures**

**Bacterial Strains and Plasmids**--E. coli C600 harboring the plasmid pDU1003 was used for the cloning of merC. This plasmid contains the mer operon from plasmid R100 cloned into pBR322 (3). The strain was a gift from Dr. S. Silver, University of Illinois at Chicago. Plasmids generated during cloning procedures were first introduced into E. coli strains K12/Tc or JM83. The merC gene was introduced into the vectors sequences deduced from DNA sequences (7–9), combined with minicell expression experiments (10, 11), suggested that MerP is a periplasmic protein, and MerT and MerC are integral membrane proteins. This arrangement is reminiscent of transport systems involving periplasmic binding proteins, which bind ligands such as maltose and histidine in the periplasmic space and then interact with one or more integral membrane transport protein(s) (for reviews, see Refs. 12–14). Such observations led to the proposal (15) that mercuric ions cross the outer membrane, bind to MerP, and are transported across the inner membrane by MerT/MerC to cytoplasmic mercuric reductase (MerA). Upon reduction of mercuric ion, metallic mercury diffuses out of the cell, and is removed from the environment of the cell by virtue of its volatility. Throughout this transport process it has been postulated that mercuric ion is always bound to pairs of cysteines on the different transport proteins: data supporting the importance of thiols has been reported for MerP and MerT (16, 17).

A number of features of the original model have been confirmed by studying resistance and/or Hg$^{2+}$ uptake in genetically manipulated strains, but conflicting data have been reported on the role of the merC gene product. Deletions of the genes merT, merC, and/or merP of the Tn21-encoded system showed that merT and merP were essential for full resistance (16, 18, 19), but that merC could be deleted without any effect on resistance, at least as measured (18). However, in Thiobacillus ferrooxidans there is no gene corresponding to merT, but rather one that is similar to merC (55% homology at the amino acid level) (20). Studies of Escherichia coli expressing merC from T. ferrooxidans indicated that MerC was involved in the uptake of mercuric ions (21, 22). In the light of these results, it would be curious if MerC of the Tn21-encoded system were merely a non-functional bystander.

The ultimate goal of the work in our laboratories is to understand how the three proteins MerP, MerC, and MerT interact with Hg$^{2+}$ and each other, and how this interaction brings about the translocation of Hg$^{2+}$ to mercuric reductase inside the cell. MerP has already been quite well characterized by ourselves and others (23–26), but biochemical information about MerT and MerC is lacking. As with other bacterial membrane transport systems, it will be necessary to purify the separate components and reconstitute them in artificial membranes to understand the complete mechanism of Hg$^{2+}$ detoxification. This paper describes the purification and partial characterization of Tn21-encoded MerC, which appears to be involved in mercuric ion uptake and binding.

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§ To whom correspondence should be addressed. Tel.: 46-90-786-6974; Fax: 46-90-786-7661; E-mail: Lena@chem.umu.se.
pCA (27) or pET3d (28). The vector pET3d containing the merC insert on an NcoI fragment will be referred to as pPWO200. E. coli BL21(DE3)pLysS (29) was used for overexpression of merC from the T7 promoter of pET3d.

Determination of Vesicle Uptake—E. coli BL21(DE3)pLysS harboring either pPWO200 (expressing merC) or pET3d (vector control) were grown and induced using IPTG as described above. E. coli BL21(DE3)pLysS were transformed with the plasmid pDU1003 and grown on LA plates containing chloramphenicol (25 μg/ml) and tetracycline (10 μg/ml). Single colonies were suspended in LB and replated: after overnight growth, colonies from one plate were suspended in 400 ml of LB broth containing tetracycline (10 μg/ml) and grown to OD600 = 0.9. At this point expression from the mer operon was induced by the addition of Hg2+ (20 μM). Since Hg2+ is volatilized during further growth, another addition of Hg2+ (10 μM) was made after 1 h. Samples of cells were harvested before induction and at 20 and 80 min after the start of induction.

Vesicles were prepared according to a method based on that described by Kaback (31). Thus, the procedure of Witholt et al. (32) was used for making spheroplasts, followed by vesicle preparation described by Kim et al. (33), except the preparation buffer was Tris-Cl, pH 8.0, instead of 7.5. The relative concentrations of vesicles were estimated by measuring the optical density at 550 nm. The relative amounts of MerC present in vesicles were estimated by scanning a polyacrylamide SDS gel electrophoresis sample buffer for 30 min before application to gels. Protein concentrations were determined using the bicinchoninic acid assay, as described by Smith et al. (35). Amino-terminal sequencing was performed using a P-1 Olsyn, Dept. of Medical Chemistry, University of Colorado.

The number of accessible thiol groups in purified MerC was estimated using 5,5′-dithiobis(2-nitrobenzoic acid) according to previously described methods (36). Determinations were carried out in 0.1 M sodium potassium phosphate buffer, pH 7.27, 1 mM EDTA, and in this buffer containing guanidine hydrochloride (5.8 M). 0.07 mM Tris-Cl, pH 8.0, 1 mM EDTA, was used when the determination was carried out in the presence of sodium dodecyl sulfate (2%). Excess cysteine in preparation buffer was removed by gel filtration through a small Sephadex G-25 column, equilibrated in 25 mM Tris-Cl, pH 8.0, containing 75 mM NaCl and 0.3% Triton X-100. Thiol determinations were also carried out on samples of MerC that had been reduced using dithiothreitol: excess reducing agent was removed by Sephadex G-25 chromatography in 25 mM MOPS buffer, pH 7.4, containing 75 mM NaCl, 0.3% Triton X-100, and 1 mM EDTA.

Hg2+ Uptake Assays—Uptake of Hg2+ by vesicles was measured by following the incorporation of radioactively labeled Hg2+. These assays involved incubation of vesicles in 50 mM Tris-Cl buffer, pH 7.3 or 8.0, for 1 min at room temperature. Hg2+ and cysteine in a 1:4 ratio, also containing 0.5 μM Hg2+, were then added. At various times, samples of 0.5 or 1.0 ml were loaded with a buffer (0.45 μM) and washed with 1.0 ml of 50 mM Tris-Cl, pH 7.3. The filters were counted in a Piconorm 4000 counter (4000, LKB-Well, Bromma, Sweden) in a counter 1214 Rackbeta. Approximately 30% of the vesicle protein typically washed through the 0.45-μm filter. No adjustment was routinely made for this. When transport was measured in the presence of thiol-modifying reagents, vesicles were preincubated for 10 min or 1 h at room temperature, in the dark with NEM, benzophenone-4-maleimide, fluorescein-5-maleimide (1 mM), or iodoacetamide/iodoacetate (10 mM). Blocking Cysteine with Hg2+—Hg2+ was preincubated with vesicles for 1 h at room temperature, and the vesicles were then washed three times and added to a Tris-Cl buffer, pH 7.3, with Hg2+ and cysteine, in a ratio of 1:4. The substrate solution was mixed 1:1 with purified MerC in a Microsep concentrator, cutoff 3000 Da, which was centrifuged for 15 min at 4400 × g. The concentrations of Hg2+ in the upper and lower reservoir were measured and the amount bound to the protein was calculated using the method previously described (28). Purified MerC used in the assay was first preincubated with a 30-fold excess of DTT for

1 The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; MOPS, 2-(N-morpholino)propanesulfonic acid; NEM, N-ethylmaleimide; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
RESULTS

Cloning and Overexpression of MerC—The gene merC was introduced into the T7 promoter-based expression plasmid, pET3d, and expressed in E. coli BL21(DE3)pLysS, as described under “Experimental Procedures.” By running samples of the insoluble fractions of cells harvested from uninduced and induced cultures on polyacylamide gels, it was possible to identify a band corresponding to MerC (Fig. 1, lanes 1 and 2). Compared with the molecular mass standards, MerC migrated at a molecular mass corresponding to 15 kDa, which agrees well with that (14.8 kDa) predicted from the amino acid sequence.

Uptake of Mercuric Ions by Vesicles and Inhibition by Thiol-specific Reagents—Vesicles were prepared from E. coli BL21(DE3)pLysS harboring the merC expression plasmid, pPOW200, or the vector control, pET3a. Uptake of radioactively labeled mercuric ions by these vesicles was measured in the presence of a 4-fold excess of cysteine to minimize nonspecific interactions (Fig. 2A). There is clearly a difference between vesicles containing MerC and the control lacking MerC. In the experiment shown, uptake in MerC vesicles was approximately 6 times higher than in the pET3a vesicles, and was generally between 5- and 8-fold higher in different experiments.

In the experiments described above the total concentration of Hg\(^{2+}\) was 1 \(\mu\)M, while the amount taken up by the vesicles was 60%, or 11 nmol of Hg\(^{2+}\)/mg of protein. Considering that up to 30% of the vesicles were typically lost in the filtration process, it is possible that all of the Hg\(^{2+}\) was taken up and therefore may have been limiting. However, when the concentration of Hg\(^{2+}\) was increased to 10 \(\mu\)M, the total amount of Hg\(^{2+}\) taken up by the vesicles was the same (data not shown), so this does not appear to be the case.

Since MerC contains two pairs of cysteine residues, Cys\(^{22,23}\)-Cys\(^{30,31}\) and Cys\(^{127,128}\)-Cys\(^{132,133}\), and cysteines have high affinity for mercuric ions, the effects on Hg\(^{2+}\) uptake of the membrane-permeable thiol-modifying reagent, N-ethylmaleimide (NEM), were also examined. As is shown in Fig. 2A, uptake of Hg\(^{2+}\) was completely inhibited by preincubation of the vesicles with NEM (1 mM), indicating that cysteine residues are indeed important for the observed uptake. By contrast, inhibition by the hydrophilic thiol modifying reagents, iodoacetate and iodoacetamide (10 mM), was much less complete in the same time period (Fig. 2B). Since NEM is membrane permeable, these results suggested that the essential cysteines are not accessible on the periplasmic side of the membrane.

Thiol accessibility has been probed in other membrane proteins using hydrophobic benzophenone-4-maleimide and hydrophilic fluorescein-5-maleimide to distinguish between surface-accessible and thiol groups buried in the membrane (37). The effects of these reagents on uptake of Hg\(^{2+}\) by MerC-containing and control vesicles are shown in Fig. 3. While the hydrophilic reagent inhibited the uptake in MerC and control vesicles by approximately 30%, practically all uptake was abolished in the MerC vesicles treated with the hydrophobic reagent. The hydrophobic reagent will pass into the membrane and thus modify cysteine residues located in the membrane, or close to it on the cytoplasmic side. These results strongly suggest that the essential cysteine residues of MerC are not accessible on the periplasmic surface.

Uptake by Vesicles with Varying Levels of MerC—Although MerC was clearly responsible for Hg\(^{2+}\) uptake, it was not clear whether the uptake exemplified by Fig. 2 represents transport to the vesicle interior or whether it represents binding to the overexpressed MerC located in the cell membrane. If it is the former, the level of uptake should be independent of the
amount of MerC in the membrane, whereas in the case of binding, the observed uptake should depend on the amount of MerC present. To prepare vesicles with varying levels of MerC, samples of E. coli BL21(DE3)pLysS harboring pPOW200 were harvested just before induction (t = 0), and at 30, 60, and 120 min, respectively, after the addition of IPTG. Vesicles were prepared and samples of them were run on an SDS-polyacrylamide gel to confirm the amount of MerC present at different time points: the amount of MerC increases with the induction time (Fig. 4A). The protein migrating at the position of MerC in the uninduced sample (Fig. 4A, lane 2) may either be a normal E. coli protein, since it is present even in a strain lacking merC altogether, or it may represent the basal expression level of MerC (29). Laser densitometry of the gel shown in Fig. 4 revealed that MerC present in vesicles from cells induced for 0, 30, 60, or 120 min represented 1, 6, 9, and 8% of total protein, respectively.

Results of Hg2⁺ uptake assays using these vesicles are shown in Fig. 4B. The vesicles were diluted for the uptake assay so that the relative concentration of vesicles, as judged from the OD at 550 nm, was the same. From these data it is clear that the uptake level is dependent on the amount of MerC present in the vesicles. This indicates that Hg2⁺ remains bound to MerC, rather than being released into the interior of the vesicle. The total amount of protein was measured and the amount of MerC in picomoles was calculated using the data from the gel shown to estimate mole of Hg2⁺ bound per mol of MerC. This value varied between 1.3 and 2.6 Hg2⁺ per MerC, regardless of whether the total concentration of Hg2⁺ was 1 or 10 μM, and this too is consistent with uptake and binding to MerC rather than uptake and release.

Influence on Hg2⁺ Uptake of Electrochemical Gradient, Osmotic Pressure, and Detergent—To probe the nature of Hg2⁺ uptake further, the ability of MerC-containing vesicles to take up mercuric ions under other conditions was examined. First, MerC vesicles were preincubated with 20 mM potassium ascorbate, pH 6.8, and 10 μM phenazine methosulfate in 50 mM potassium phosphate buffer, pH 7.3. These conditions have been shown to drive numerous other periplasmic protein-dependent transport systems by creating an electrochemical gradient across the membrane and/or generating ATP inside vesicles (13, 38). Preincubation under these conditions did not increase the observed uptake of Hg2⁺ (Fig. 5). Vesicles were also preincubated in Tris-Cl buffer, pH 7.3, containing sucrose (0–1 M). Under conditions of increased osmotic pressure one would expect to see decreased uptake, if Hg2⁺ were transported across the membrane and released into the interior, as has been observed for histidine uptake (38). However, there was no effect of increasing sucrose concentrations on the uptake of mercuric ions (Fig. 5). Finally, vesicles were also preincubated for 1 h with 0.05% Triton X-100. This treatment should render the vesicles leaky, so that any substance released into the vesicle interior could leak out again. The Triton X-100-treated vesicles exhibited reduced Hg2⁺ uptake, to about 60% of that shown by vesicles not treated with Triton X-100, but still 4 times higher uptake than the control vesicles (Fig. 5). All these data provide further evidence that Hg2⁺ is not transported across the membrane and released, but rather that the ions remain bound to MerC in the vesicle membrane.

Purification of MerC—MerC was purified both in the presence and absence of cysteine, as described under “Experimental Procedures.” After cell breakage and ultracentrifugation MerC was found in the membrane fraction (Fig. 1, lanes 3 and 4), so initially several different detergents were tested for their ability to solubilize it. Tween 20, n-octyl glucoside, CHAPS, and deoxycholate all solubilized MerC poorly (data not shown), whereas both Nonidet P-40 and Triton X-100 gave good and relatively selective solubilization (Fig. 1, lanes 5 and 6). N-
phosphate buffer, pH 7.3. Vesicles were also preincubated with either potassium phosphate, pH 7.3. The amount taken up after 15 min is described in the legend to Fig. 2, except that the buffer was 50 mM or the other additives indicated. Uptake of Hg\(^{2+}\) Vesicles were preincubated for 1 min in 50 mM potassium disulfide-bonded multimer formation, samples of purified MerC were treated with SDS gel mixture in the presence or absence of MerC were submitted for determination of the amino-terminal sequence, and the result, Gly-Leu-... 10 \(\mu\)M phenazine methosulfate and 20 mM potassium ascorbate, pH 6.8, or the other additives indicated. Uptake of Hg\(^{2+}\) was measured as described in the legend to Fig. 2, except that the buffer was 50 mM potassium phosphate, pH 7.3. The amount taken up after 15 min is shown.

Dodecyl-\(\beta\)-d-maltoside also solubilized MerC, but was not as selective. Triton X-100 was therefore chosen as the most suitable detergent. Since extraction using Triton X-100 was done at a high salt concentration, this solution had to be diluted before application to the DEAE column. Following this column, a gel filtration step was performed, after which MerC appeared to be essentially pure, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 1). The yield from 2 liters of culture was approximately 30 mg.

A sample of purified protein was submitted for determination of the amino-terminal sequence, and the result, Gly-Leu-Met-Thr-Arg, was as expected from the DNA sequence with the initial Met cleaved off. No contaminating proteins were detected during the sequencing cycles.

**Properties of Purified MerC**—To examine the possibility of disulfide-bonded multimer formation, samples of purified MerC were treated with SDS gel mixture in the presence or absence of \(\beta\)-mercaptoethanol. From the gel shown in Fig. 6 it is clear that under nonreducing conditions the protein exists as a dimer as well as a monomer (lanes 1 and 2) while under reducing conditions almost none of the MerC is dimeric (lanes 5 and 6). MerC that had been purified in the absence of the reducing agent, cysteine, had one-third of MerC in the form of a dimer as well as a monomer (lanes 1 and 2, Fig. 6). When the samples were incubated with DTT for 1 h at room temperature (see below), and freed of excess DTT by gel filtration, most of the MerC ran at the position of the monomer (data not shown). Taken together, these data indicate that some MerC as purified is a disulfide-bonded dimer. The difference in the proportions of dimer and monomer from the different preparations indicates that the cysteines participating in the dimer formation are sensitive to oxidation. Apparently the presence of cysteine facilitates the formation of intermolecular disulfide bonds, perhaps via intermediate formation of a mixed disulfide with one or more cysteine residues of MerC.

Since oxidation of MerC during purification might be the cause of dimerization, the existence of disulfide-bonded dimer was also examined in vesicle samples. Vesicles were treated with SDS sample mixture with or without \(\beta\)-mercaptoethanol, and run on an SDS-polyacrylamide gel (Fig. 6, lanes 3, 4, 7, and 8). As can be seen, MerC exists partially as a dimer in the vesicle preparations as well, but the ratio of monomer to dimer is considerably higher, approximately 4:1. Therefore, handling during purification appears to exacerbate dimer formation. We cannot exclude the possibility that the dimers are formed in vesicles because there is so much MerC present in the membrane in this overexpressing system, and that MerC expressed at wild-type low levels would mainly exist as a monomer. However, to detect MerC monomers and dimers at the lower expression levels in the native strains, it will be necessary to obtain MerC-specific antibodies.

**Thiol Group Determinations**—The amino acid sequence predicts the existence of four cysteines in MerC. The number of accessible thiol groups in MerC purified in the presence and absence of cysteine was therefore examined using 5,5'-dithiobis(2-nitrobenzoic acid). No thiol groups of MerC reacted with 5,5'-dithiobis(2-nitrobenzoic acid) in native, guanidinium hydrochloride (5.8 M), or SDS (2%)-treated samples. After incubation at room temperature for 1 h with a 20-fold excess of DTT, followed by removal of excess reducing agent, MerC preparations showed 1.7–1.9 thiol groups/protein. SDS-polyacrylamide gel electrophoresis analysis (see above) indicated that these samples were mostly monomeric. Therefore, the other two thiols may either be inaccessible to DTT, or to the bulky DNTB molecule.

**Binding of Mercuric Ions to Purified MerC**—Data collected using vesicles strongly suggested that uptake of Hg\(^{2+}\) represents binding to MerC in the membrane. Therefore the binding properties of purified MerC were studied using the same assay that was developed for binding studies of MerP (Fig. 7). These binding studies were performed in the presence of a cysteine to Hg\(^{2+}\) ratio of 4:1, so that nonspecific binding like that observed with MerP in the absence of cysteine should be avoided (23). MerC that had been prereduced with DTT can clearly bind Hg\(^{2+}\) (Fig. 7). However, in contrast to the binding data obtained with MerP, the data for MerC could not be fitted well to an equation describing binding to one site. Thus it seems as if either binding is more complex than simple binding to one site,

**FIG. 5. Dependence of Hg\(^{2+}\) uptake by vesicles on various additives.** Vesicles were preincubated for 1 min in 50 mM potassium phosphate buffer, pH 7.3. Vesicles were also preincubated with either 10 \(\mu\)M phenazine methosulfate and 20 mM potassium ascorbate, pH 6.8, or the other additives indicated. Uptake of Hg\(^{2+}\) was measured as described in the legend to Fig. 2, except that the buffer was 50 mM potassium phosphate, pH 7.3. The amount taken up after 15 min is shown.

**FIG. 6. SDS-polyacrylamide gel electrophoresis of purified and vesicular MerC under oxidizing or reducing conditions.** 4–20% gradient SDS-polyacrylamide gel. Samples in lanes 1–4 were treated with SDS sample mixture without \(\beta\)-mercaptoethanol, and samples in lanes 5–8 were treated with SDS mixture containing \(\beta\)-mercaptoethanol. Lanes 1 and 5, MerC purified in the absence of cysteine; lanes 2 and 6, MerC purified in the presence of cysteine; lanes 3 and 7, vesicles from cells expressing MerC; lanes 4 and 8, vesicles from cells containing the pET control vector (no MerC expressed). The arrow shows the location of the dimer of MerC.
or that the method is not suitable for measurement with MerC in Triton X-100 micelles.

**Prediction of MerC Topology**—The TopPred II (39) program was used to predict the topology of MerC. This program uses an algorithm that takes into account the hydrophobicity of amino acid residues as well as the “positive inside” rule (40), which has been found to be very effective in predicting the topology of bacterial inner membrane proteins. The predicted model for MerC is shown in Fig. 8, where the amino acids are numbered assuming that the initial methionine has been cleaved off. This model places the two cysteine residues 22 and 25 at the amino-terminal end of the first α-helix, while the remaining two cysteines, 127 and 132, are in a loop on the cytoplasmic side. Thus all four cysteine residues are predicted to be on or near the cytoplasmic side of the inner membrane which would mean that there are no cysteine residues on the periplasmic side that can pick up Hg²⁺. This prediction is in agreement with the data using thiol modifying agents. No other amino acid residues, such as histidine, which have high affinity for Hg²⁺, are predicted by this model to be located on the periplasmic side.

**Effects of MerP on Uptake**—In other systems with periplasmic binding proteins, the transported compound is not transferred across the inner membrane in the absence of the binding protein (12–14). Therefore, the effects of added MerP on the uptake of Hg²⁺ by MerC-containing vesicles were examined. As can be seen in Fig. 9, 30 μM MerP inhibited Hg²⁺ uptake to 6.4% of the uptake observed in the absence of MerP. Considering that the Kᵣ for MerP is 2.8 μM Hg²⁺, and the total concentration of Hg²⁺ in this experiment was only 1 μM, it appears that Hg²⁺ remained associated with MerP rather than being transferred to MerC. Therefore uptake of Hg²⁺ was also measured in the presence of only 0.3 μM MerP and was found to be 92% of that observed in the absence of MerP, which is within the margins of error. Thus, MerC has a high enough affinity for Hg²⁺ so that in the periplasm at least some Hg²⁺ can be transferred to MerC even in the presence of MerP. However, MerP is not necessary for MerC-mediated mercuric uptake, and depending on the relative concentrations may in fact inhibit it.

**Hg²⁺ Uptake in Vesicles Containing MerT and MerC**—Although MerP is not necessary for MerC-mediated Hg²⁺ uptake, perhaps MerT plays a crucial role since in the Tn21-encoded system membrane-bound MerT and MerC are produced together. To test this question, vesicles were prepared from E. coli BL21/DE3pLysS harboring the plasmid pDU1003, which encodes the complete Hg²⁺-inducible mer operon (3). Hg²⁺ uptake was measured in vesicles prepared from cells induced for 0, 20, and 80 min, respectively. As can be seen in Fig. 10, uptake in vesicles prepared from induced *versus* uninduced cells was approximately 3.8-fold higher. Uptake was also measured in the presence of 100 mM NaCl, since it has been suggested that Hg²⁺ may be co-transported with a sodium gradient (41). There was no difference between uptake in the absence or presence of NaCl (Fig. 10). Preincubation with phenazine methosulfate and ascorbate did not significantly change the measured Hg²⁺ uptake, as was observed with vesicles containing only MerC (see above), nor was uptake increased in the presence of MerP (data not shown). The total amounts of Hg²⁺ taken up by these vesicles were 1.26, 4.78, and 4.00 nmol/mg of protein, for the vesicles induced for 0, 20, and 80 min, respectively. These values are approximately 3-fold lower than the corresponding values for the MerC-containing vesicles. It is important to note, therefore, that the levels of MerC and MerT present in these vesicles are much lower than in the vesicles from the cells overexpressing MerC (data not shown). Although the total uptake of Hg²⁺ in these vesicles was approximately 3-fold lower than that observed in the vesicles containing only MerC, the difference between uptake in vesicles prepared from induced *versus* uninduced cells is quite similar. We have also obtained similar results using vesicles...
prepared from cells overexpressing only MerT. Therefore, the results obtained using the vesicles containing only MerC do not appear to reflect the fact that MerT is absent.

**DISCUSSION**

Bacterial detoxification of mercuric ion has been postulated to include a mercuric ion transport system comprising the periplasmic protein, MerP, and the membrane proteins, MerC and/or MerT. In the Tn501-encoded system, which lacks merC, deletion analysis indicated that MerP and MerT were both necessary for full resistance (19), but MerT alone was apparently sufficient for mercuric ion transport (16). On the other hand, studies of MerC from *T. ferrooxidans*, which lacks both MerT and MerP, link Hg$^{2+}$ uptake by whole cells to the presence of MerC (21, 22). Therefore, it appears that either MerC or MerT can facilitate mercuric ion uptake, but in neither case has mercuric ion transport been characterized at the biochemical level.

The Tn21-encoded mercuric ion resistance operon is complicated by the existence of both merC and merT. In a study of the roles of the different Hg$^{2+}$-resistance proteins in the Tn21 system, construction of deletion mutants showed that both merT and merP were necessary for full resistance to 50 μM Hg$^{2+}$: deletion of merT had a greater impact than deletion of merP (18). Mutants lacking only merC still showed uptake of Hg$^{2+}$, but upon deletion of only merT, uptake of Hg$^{2+}$ ceased suggesting that only merT is essential. However, since these studies used single-copy plasmids and protein expression was not quantitated, it is possible that the levels of MerC expression were not high enough in these strains to detect uptake. These data thus do not rule out the involvement of MerC in mercuric ion uptake in the Tn21-encoded system, a role which might be expected based on the observation that merC is the only membrane protein-encoding gene present in some mer operons.

Our data clearly show that Tn21-encoded MerC mediates specific Hg$^{2+}$ binding. None of the collected evidence was consistent with a role for MerC as a channel protein that allows Hg$^{2+}$ to flow unrestricted into the cytoplasm. Rather, the lack of energy dependence, the fact that uptake was unaffected by osmotic pressure, and the observation that the amount of MerC present in the vesicles affected the level of Hg$^{2+}$ uptake, all indicate that mercuric ion is taken up and remains bound to MerC.

Little attempt has previously been made to distinguish between the flow of Hg$^{2+}$ into the cytoplasm of resistant bacteria and the type of controlled binding we observed for MerC. However, nothing in previously published data supports the idea that MerC or MerT function as channel proteins. For example, Nakahara et al. (6) found that Hg$^{2+}$ uptake was not inhibited by toluene treatment, detergent treatment, or even heat treatment. These observations are not consistent with the existence of Hg$^{2+}$-conducting channel proteins, but support the idea that the membrane proteins bind Hg$^{2+}$. In studies where the amounts of Hg$^{2+}$ taken up by cells have been quantitated, the levels of mercuric ions that get incorporated are all low relative to appropriate controls. Thus, the difference in Hg$^{2+}$ uptake between induced and uninduced cells harboring the Tn21-encoded uptake system was approximately 3-fold (6, 18), when the total concentration of Hg$^{2+}$ was 2 μM. In two different studies of uptake in *Thiobacillus*, a 2- (21) or 5-fold (22) difference between induced and uninduced cells was reported where the total concentrations of Hg$^{2+}$ were 5 and 2.5 μM, respectively. These differences in uptake levels are similar to those observed in this study (Fig. 2).

A crucial point is therefore where the Hg$^{2+}$-binding site is located in MerC. According to the predicted membrane topology of MerC, two pairs of cysteine residues are both located on the cytoplasmic side of, or in, the membrane. Inhibition of MerC-dependent uptake by thiol reagents supports the notion that cysteine residues are important. Uptake of Hg$^{2+}$-complexed to cysteine was completely inhibited in the presence of the membrane-permeable sulphydryl reagent, NEM (Fig. 2), and the hydrophobic benzophenone-4-maleimide (Fig. 3). By contrast, there was little inhibition observed using the hydrophilic reagent fluorescein-5-maleimide (Fig. 3) or 10-fold higher concentrations of the hydrophilic thiol reagents, iodoacetate and iodoacacetamide (Fig. 2B). These data are all consistent with the essential thiol being located either within the membrane bilayer, or close to it on the cytoplasmic side, as predicted by the computer analysis (Fig. 8). These results therefore rule out the possibility that MerC simply binds nonspecifically to accessible thiols on the periplasmic surface, and imply that MerC can mediate transport through the bilayer: however, in the absence of a driving force (see below), Hg$^{2+}$ remains bound to MerC.

The amino acid sequence GMXXCXXC has been proposed to be a consensus heavy metal binding motif, and is present in MerP, where it has been correlated with specific binding of Hg$^{2+}$ (24, 26), as well as in proteins transporting copper and cadmium (42). This motif is also present in the aminoterminal region of mercuric reductase but does not appear to affect the enzymatic activity (43); it is not known whether it mediates Hg$^{2+}$ binding. The presence of a nearly identical sequence in MerC around Cys$^{22}$ and Cys$^{29}$ suggests that these residues are the ones involved in mercuric ion binding. Interestingly, these residues are predicted to lie near or in the bilayer. In contrast, MerT does not have this sequence, suggesting that if it binds Hg$^{2+}$, it may do so in a different mode possibly involving MerT multimers as suggested by Brown and co-workers (16, 17). Indeed, it is difficult to imagine how the amino-terminal cysteine pair could bind Hg$^{2+}$ in a MerT monomer, since the two residues are adjacent to each other in a proposed transmembrane helix.

Most membrane transport proteins are rather large: the maltose transporter appears to consist of two proteins, with 6 and 8 α-helices each (14) and the lac permease has 12 α-helices (44), whereas MerC and MerT only have four and three (16) predicted α-helices, respectively. It is, of course, possible that either or both of these proteins forms dimers or other mul-

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2 L. Sahlman and J. Powloski, unpublished results.
timers in the membrane, and that these are the functional units. Our preparations of MerC show that there is indeed some dimerization due to intermolecular disulfide bonding, but we cannot rule out the possibility that this results from the high expression levels achieved in the strains studied. In wild-type cells where MerT is also present, MerC molecules might interact with MerT, thus limiting the interaction between MerC molecules. Since we detected no substantial differences in Hg\(^{2+}\) uptake behavior when both proteins were expressed, present evidence indicates that physical interaction between MerC and MerT is not essential for Hg\(^{2+}\) uptake by MerC. The question of the quaternary structures of MerC and MerT needs to be more fully examined in the future, however.

An important observation was that MerP appears to have no required role in transfer of Hg\(^{2+}\) across the membrane. Uptake in MerC-containing vesicles was not increased in the presence of MerP, nor was uptake by the MerC-MerT containing vesicles from cells harboring the complete operon increased in the presence of MerP. This is in marked contrast to periplasmic binding-protein dependent systems (e.g. for amino acid uptake), and conflicts with a widely accepted model for Hg\(^{2+}\) detoxification (15): in these cases the periplasmic protein initially accepts the ligand and transfers it to membrane-bound transport proteins. A reasonable conclusion is therefore that instead, MerP primarily acts as a scavenger, binding Hg\(^{2+}\) and keeping it away from other periplasmic proteins which require thiol groups for activity, as has been suggested by Summers (45). This interpretation is also in agreement with genetic data which inferred that MerP is not necessary for MerT-mediated Hg\(^{2+}\) uptake (16, 18). However, by default, MerP-bound Hg\(^{2+}\) may still be the main periplasmic source of any mercuric ion transferred to MerC and/or MerT.

How, then, does Hg\(^{2+}\) pass through the membrane, if not by a periplasmic binding protein-mediated process? Two possibilities for Hg\(^{2+}\) passage through the inner membrane are diffusion through the bilayer, and passage via MerC and/or MerT. Studies have shown that phospholipids interact with Hg\(^{2+}\) and that membranes are permeable to HgCl\(_2\) (46, 47), but no studies have been made using Hg\(^{2+}\) coupled to thiols. Since thiol ligation of Hg\(^{2+}\) tends to suppress nonspecific (weaker) interactions (e.g. Refs. 23 and 48), our experiments were always carried out in the presence of a 4-fold excess of cysteine. If transfer of this complex across the membrane occurs passively, it must be very slow since uptake by control vesicles was relatively low, and could as easily represent surface binding as transport. The observation that MerC binds mercuric ion via surface-inaccessible thiols strongly suggests that this protein has a role in actual transfer across the membrane, in addition to its binding function. If so, what drives the release of Hg\(^{2+}\) from MerC? Considering the metal-binding consensus sequence at the amino terminus of mercuric reductase, and its homology to MerP, it is likely that Hg\(^{2+}\) does indeed bind there, prior to transfer to the active site for reduction. Transfer of Hg\(^{2+}\) from MerC to the amino-terminal thiols of mercuric reductase thus may provide the “trigger” for release of mercuric ion from MerC: such a trigger is not present in the vesicles used in this study. The release of Hg\(^{2+}\) to mercuric reductase, and its reduction, would provide the driving force for transport in a bacterium harboring all of the proteins necessary for Hg\(^{2+}\) resistance.

Taking these data together, a revised model for the Hg\(^{2+}\) detoxification system encoded by Tn21 may be proposed. Of the 5 structural genes encoded by the operon, we propose that three are Hg\(^{2+}\)-binding proteins, namely MerP, MerC, and the amino-terminal region of mercuric reductase. This assertion is based on the existence of the heavy metal consensus binding sequence, GMXCXXC, in these proteins, as well as the demonstrated binding properties of MerP (24, 26) and MerC (described in this paper). With specific Hg\(^{2+}\)-binding proteins in the periplasm, inner membrane, and cytoplasm, Hg\(^{2+}\) would always be sequestered to a protein, minimizing interaction with other cellular proteins in these compartments. In the event that Hg\(^{2+}\) actually did get into the cytoplasm, it would do so via MerC (or MerT) and be intercepted by mercuric reductase. Thus, a crucial difference between this and earlier models is that although MerP/MerC/MerT participate in transport they are not designed to maximize transport of Hg\(^{2+}\) into the cell. Although MerT is clearly important for uptake in vivo and may supplement or complement MerC in the Tn21 system, further studies are required to define the molecular mechanism of MerT in this system.

If the transport proteins do not permit unrestricted flow of Hg\(^{2+}\) into the cell, why are bacteria lacking functional mercuric reductase hypersensitive to Hg\(^{2+}\)? (6)? In our experiments, vesicles are devoid of cytoplasmic proteins so there is nothing to compete with the binding sites on MerC for Hg\(^{2+}\). However, although Hg\(^{2+}\) binds tightly to thiols, there is also rapid equilibrium with other thiols (48), so in a wild-type cell it is possible that enough mercuric ions are leached off MerC to produce the toxic effects seen as bacteria are growing. However, when mercuric reductase is present a specific transfer mechanism, followed by reduction of Hg\(^{2+}\), may protect cytoplasmic proteins from interaction with mercuric ions. Perhaps the biggest unknown question is how Hg\(^{2+}\) can be released from MerC to mercuric reductase in the cytoplasm, and that question is currently under investigation in our laboratories.

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