EmrE is a small, 12-kDa, highly polyspecific antiporter, which exchanges hydrogen ions with aromatic cations such as methyl viologen. EmrE-mediated transport is inhibited by the sulphhydryl-reactive reagent 4-(chloromercuri)benzoic acid (PCMB) but not by a variety of other sulphhydryl reagents. This differential effect is due to the fact that the organic mercurial is a substrate of the transporter and can reach domains otherwise inaccessible to the different reagents. To find out which of the three cysteine residues in EmrE is reacting with PCMB, each was replaced with serine and it was shown that none of them is essential for transport activity. A protein completely devoid of Cys residues (CL) is also capable of substrate accumulation albeit at a slower rate. Mutated proteins in which only one of the native cysteines was left whereas the other changed to serine were also constructed. The use of these proteins demonstrated that two of the three Cys in EmrE, Cys-41 and Cys-95, but not Cys-39, react with PCMB. A related mercurial, 4-(chloromercuri)benzenesulphonic acid (PCMBs), is only a very poor inhibitor, probably because of the negative charge it bears. PCMBs reacts with EmrE in an asymmetric and unique way. It reacts with the mutant bearing a single Cys residue in position 95 (CL-C95) only when the reagent is present in the outside face of the membrane and with the mutant CL-C41 only when allowed to permeate to the cell interior; as expected, it does not react with the mutant protein bearing a single Cys at position 39 (CL-C39). It is concluded that PCMB permeates through the substrate pathway of EmrE and covalently reacts with the two exposed residues, Cys-95 and Cys-41, but not with Cys-39, located on the opposite face of the helix relative to residue 41. In addition, because of the asymmetric reactivity to PCMBs, an inhibitor that does not permeate through the protein, it is concluded that Cys-41 is closer to the cytoplasmic face than Cys-95. The results demonstrate the existence of a domain accessible only to substrates and provide a unique tool for studying the substrate permeation pathway of an ion-coupled transporter.

Resistance to a wide range of cytotoxic compounds is a common phenomenon observed in many organisms throughout the evolutionary scale and probably developed in order to cope with the variety of toxic compounds that are part of the natural environment in which living cells dwell (see for example Refs. 1–3). One of the survival strategies that evolved is removal of toxic substances by transport. Multidrug transporters are the proteins usually responsible for performing this task and have been found in many organisms, from bacteria to man (4–9). They actively remove a wide variety of substrates in an energy-dependent process and thereby decrease the concentration of the offending compounds near their target. A great diversity of multidrug transporters are known to us today, and we can group them in several different families based on structure similarities. A unique family (Smr or MiniTEXANS) is represented by very small proteins, about 100 amino acids long, which render bacteria resistant to a variety of toxic cations (10, 11).

Two MiniTEXANS, Smr (12, 13) and EmrE (14), have been characterized, purified and reconstituted in a functional form. Both proteins catalyze H+/cation antiport in proteoliposomes reconstituted with purified transporter and behave as multidrug transporters capable of recognizing a wide range of substrates and inhibitors. In addition, EmrE has been shown to display unique properties of solubility in organic solvents such as a mixture of chloroform and methanol (14). After solubilization in the above solvent, the protein retains its ability to transport as judged from the fact that it can be reconstituted in a functional form. Using transmission FTIR (Fourier transform infrared) and oriented ATR-FTIR (attenuated total reflection FTIR) spectra, the protein was found to be a four-membered transmembrane antiparallel helical bundle. The helices in EmrE are oriented perpendicular to the lipid bilayer with a tilt angle of 27° with respect to the bilayer normal (15).

An elegant and systematic approach to the identification of residues lining an ion channel has been applied to the acetylcholine and γ-aminobutyric acid receptors and to the cystic fibrosis transmembrane conductance regulator (16–18). Each residue in putative transmembrane spanning segments is mutagenized to cysteine and the accessibility of the new cysteine to small, charged, water-soluble, sulphhydryl-specific reagents is tested. The polar reagents react only with sulphhydrals at the water accessible surface of the protein, i.e. those exposed in the channel lumen. The covalent attachment of any of these groups to a cysteine in the channel is assumed to alter the channel function. This approach, called the substituted-cysteine accessibility method, allows, therefore, identification of residues in the permeation pathway. In a variation of this method, Ag⁺, a sulphhydryl-reactive substrate of a K⁺ channel was used to study the channel exposed residues (19) and selected cysteine point mutations in residues located at or near the aqueous pore were found to confer mercurial sensitivity to a mercurial-insensitive water channel (20). In general, however, the method is limited to those proteins in which the substrate pathway is hydrophilic and accessible to molecules other than the substrate. In ion-coupled transporters, on the other hand, at least part of the permeability pathway must be reachable only by substrates or inhibitors. Yan and Maloney have probed a bacterial membrane antiporter that exchanges glucose 6-phos-
acids; Tricine, N\textsubscript{fonic acid; IAA, iodoacetic acid; MTSEA, methanethiosulfonate ethyl-sulfonic acid (PCMBS), a hydrophilic and impermeant phosphate and inorganic phosphate with 4-(chloromercuri)benzene-sulfonic acid (PCMB),\textsuperscript{3} a hydrophilic and impermeant sulfhydryl-reactive agent presumably resembling the substrate in volume and charge (21, 22). They showed that transmembrane-spanning segment 7 is an \( \alpha \)-helix whose central portion is accessible to PCMB.

In this report we show that the three native cysteine residues in EmrE (Fig. 1) are inaccessible to either small hydrophilic reagents, such as the negatively charged iodoacetic acid (IAA) and the positively charged methanethiosulfonate ethyl-ammonium (MTSEA), or to hydrophobic ones such as N-ethylmaleimide (NEM). However, a sulfhydryl-reactive substrate such as PCMB inactivates transport of the wild type protein because it is a substrate of the transporter and can reach domains otherwise inaccessible to the other reagents. Each of the cysteine residues in EmrE was replaced with serine, and it was shown that none of them is essential for transport activity. A protein completely devoid of Cys residues is also capable of substrate accumulation, albeit at a slower rate, and it was used to generate mutants with a single Cys residue at each of the Cys positions in the wild type protein. Using these mutant proteins it was found that two of the three Cys in EmrE, Cys-41 and Cys-95, but not Cys-39, react with PCMB. PCMB, which is a very weak inhibitor because of the negative charge it bears, reacts with EmrE in an asymmetric and unique way; it reacts with Cys-95 from the outside face of the membrane and Cys-41 from the inside.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Bacterial Strains and Plasmids—}\textit{Escherichia coli} JM 109 (23) and plasmid pK 56 (24) are used throughout this work. In plasmid pK 56, EmrE is cloned into EcoRI and HindIII sites of pKK223-3 (Pharmacia Biotech Inc.).

\textbf{Mutagenesis—}Mutants were obtained by polymerase chain reaction mutagenesis using the overlap extension procedure described by Ho et al. (24). For each mutation a set of two overlapping oligonucleotide primers containing the desired mutation were constructed. The outside primers were those used for the wild type EmrE (24).

\textbf{Mutagenic oligonucleotides} were prepared incorporating a unique restriction site to facilitate mutant identification, and in several cases this required an additional conservative mutation that did not affect EmrE expression. Mutated DNA was identified by the acquisition of the new restriction site and sequenced to ensure that no other mutations occurred during the amplification process (Sequenase version 2.0, U. S. Biochemical Corp.).

The primer 5\textsuperscript{\textprime}GTTACGATCTTTCTTATTGG and with a unique Sau3AI site and a conservative change in Thr-36 and Ile-37 were used to obtain the C39S variant when using wild type DNA as template or CL-C41 when using C95S as DNA template; 5\textsuperscript{\textprime}GTTACAGATTGTTGTCGAAGCATTGCATTTGG with a unique CfoI site yielded CL-C41 when using wild type DNA as template; 5\textsuperscript{\textprime}GATGGATTGTTGCAAGCATTGCATTTGG with a unique CfoI site yielded CL-C95 when using wild type DNA as template; 5\textsuperscript{\textprime}GTTACAGATTGTTGTCGAAGCATTGCATTTGG with a unique CfoI site was used to give CL, when using C95S as DNA template and CL-C95 when using wild type as DNA template.

\textbf{Resistance to Toxic Compounds—}For testing resistance to toxic compounds, cells were grown at 37°C in LB-Amp medium with different concentrations of the compound and \( A_{600nm} \) was checked.

\textbf{Expression, Purification, and Reconstitution of EmrE—}\textit{E. coli} JM 109 transformed with the appropriate plasmid was grown in minimal medium \( A \) with 0.5% glycerol and thiamine and ampicillin as above. When the culture reached an \( A_{600} \) of 0.9, isopropyl \( \beta \)-\textit{D}-thiogalactoside was added to 0.5 \( \text{mM} \); 2 h later, the cells were chilled on ice and harvested by centrifugation. Membranes were prepared by disrupting the cells using a French pressure procedure (25), except that the buffer was used 30 mM Tris-Cl, pH 7.5, 250 mM sucrose, 150 mM choline chloride, 0.5 mM dithiothreitol, 2.5 mM MgSO\textsubscript{4}, and 15 \( \mu \)M DNase I. After ultracentrifugation membranes were resuspended at 10 mg of protein/ml, frozen in liquid air, and stored at −70°C. EmrE was extracted essentially as described (24) from 2.5 ml of membranes (10 mg of protein/ml) with 37 ml of chloroform/methanol (1:1), incubated 20 min on ice. For phase separation, 7.5 ml of H\textsubscript{2}O were added and the suspension was centrifuged. The upper phase was removed, and the lower phase with the enriched protein was stored at −70°C. For analysis in SDS-polyacrylamide gel electrophoresis, a sample was dried, resuspended in sample buffer, and analyzed in 16% gels as described (26).

For reconstitution, 4–9 \( \mu \)g of extracted protein was mixed with 18 \( \mu \)l of \textit{E. coli} phospholipids (50 mg/ml), 750 \( \mu \)l of chloroform/methanol (1:1) and 180 \( \mu \)l chloroform/methanol (1:2). The suspension was dried with argon and resuspended in a solution (60 \( \mu \)l) containing 0.18 M NH\textsubscript{4}Cl, 0.015 M Tris-HCl, pH 6.9. The suspension was kept at −70°C. Before the assay, the proteoliposome suspension was thawed and sonicated in a bath-sonicator for a few seconds until clear.

\textbf{Transport Assay—}Transport of \( ^{14}\text{C} \) methyl viologen into proteoliposomes was assayed by dilution of 3 \( \mu \)l of the ammonium chloride containing proteoliposomes into 200 \( \mu \)l of an ammonium-free medium containing 35 \( \mu \)l \( ^{14}\text{C} \) methyl viologen (90 nCi), 140 mM KCl, 10 mM Tricine, and 5 mM MgCl\textsubscript{2}, (pH 8.3). At given times, the reaction was stopped by dilution with 2 ml of the same ice-cold solution, filtering through Schneider & Schüll filters (0.2 \( \mu \)m) and washing with an additional 2-ml solution. The radioactivity on the filters was estimated by liquid scintillation. In each experiment the values obtained in a control reaction with 5 \( \mu \)M nigericin were subtracted from all experimental points. This background was between 5 and 10% of the experimental values.

\textbf{Transport with Sulphydryl Reagents—}Proteoliposomes of wild type EmrE and the various mutants (75–300 ng of protein/\( \mu \)l) reconstituted in ammonium chloride medium were preincubated for the indicated times at 22°C with different concentrations of sulphydryl reagents and diluted 60-fold into the ammonium-free medium containing \( ^{14}\text{C} \) methyl viologen as described above.

\textbf{Protein Determination—}Protein determination was done in dry samples according to Peterson (27).

\textbf{RESULTS}

\textit{EmrE} Shows Differential Sensitivity to Various Sulphydryl Reagents—To test the role of Cys residues in EmrE-mediated transport, we mutagenized each of the three cysteines in EmrE and tested the effect of a variety of sulphydryl reagents. After treatment of proteoliposomes reconstituted with highly enriched EmrE with the various reagents, transport of \( ^{14}\text{C} \) methyl viologen was assayed by dilution of ammonium chloride-loaded proteoliposomes into an ammonium-free medium. Under these conditions a pH gradient was generated, acid inside, which was utilized by EmrE to accumulate \( ^{14}\text{C} \) methyl viologen against its concentration gradient in a

\textsuperscript{3}The abbreviations used are: PCMBs, 4-(chloromercuri)benzenesulfonic acid; IAA, iodoacetic acid; MTSEA, methanethiosulfonate ethyl-ammonium; NEM, N-ethylmaleimide; PCMB, 4-(chloromercuri)benzenesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
concentrations as high as 3 mM for the above reagents had no

effect on the initial rate of pH-driven [14C]methyl viologen
accumulation. The hydrophobic neutral NEM had also no effect
on transport up to a concentration of 12 mM (only part of the
concentration range of NEM is shown in Fig. 2A). On the other
hand, a relatively more bulky compound, PCMB, inhibited
transport in a concentration-dependent way; pretreatment with
about 1 mM PCMB results in a 50% decrease in the initial
rate and practically full inhibition is detected above 1.5 mM.
This compound is also relatively hydrophobic but it bears a
positive charge (+Hg+) in proximity to the aromatic benzene
ring (Fig. 2B). In position para to the Hg, a weak carboxyl
moiety is usually ionized at neutral pH. The more hydrophilic
analogue, PCMBs, bears a sulfonate residue instead of the
carboxyl at PCMB (Fig. 2B) and is, therefore, permanently
charged; PCMBs inhibits the EmrE-mediated transport with
slightly higher potency: 50% inhibition at 0.5 mM, full-inhibi-
tion above 1.5 mM (Fig. 2A). The PCMB-inhibited protein is
completely inactive, as judged not only from its inability to
drive pH-dependent uptake. It cannot catalyze downhill ef-
flux of [14C]methyl viologen from preloaded liposomes or ex-
change of the intraliposomal substrate with an excess of unla-
bled substrate (data not shown). The inhibition by both
reagents is fully reversible after a 10-min incubation with 5 mM
dithiothreitol, as expected from the type of reaction of these two
mercurials with Cys residues (data not shown).

Since the reactivity of the other sulphydryl reagents is cer-
tainly not lower than that of PCMB and PCMBs, we tested
whether it is possible that they react but do not inhibit activity.

To test this assumption the proteoliposomes were exposed to
the various compounds prior to PCMB treatment to test for
“protection” of the inhibition site. The results of such an ex-
periment, in which wild type and several single cysteine mutant
proteins (see below for details) were exposed to the reagents,
are summarized in Table I. The results suggest that none of
the reagents, except for the mercurials, react with any of the sen-
sitive Cys: pretreatment of the proteoliposomes with 3 mM of
IAA, MTSEA, or NEM have no effect by itself on the
activity, as shown above and also do not prevent the inhibition
by PCMB.

Replacement of the Three Cysteines in EmrE by Site-directed
Mutagenesis —To identify the residue(s) that are the site of
action of PCMB and PCMBs and to test for their possible role
in the transport reaction, each of the three Cys in EmrE:
Cys-39, Cys-41, and Cys-95, was replaced with Ser and also a
strain in which all three of them were replaced (CL)2 was
obtained. The results in Fig. 3 summarize the phenotype of
cells bearing the various mutants: E. coli cells, otherwise sen-
sitive to the toxic effects of ethidium bromide (400
µg/ml; Fig. 3C), methyl viologen (0.4 mM, Fig. 3B), and acriflavine (50
µg/ml; Fig. 3C), become resistant to the above compounds when
transformed with a multicopy plasmid carrying wild type
type EmrE. Essentially the same phenotype is observed when the
cells are transformed with plasmids coding for mutants C39S,
C41S, C95S, or for CL, a completely Cys-less protein (Fig. 3).
Control cells, mock-transformed with vector only, are sensitive
to all the above compounds, while growing normally in the
presence of ampicillin (Fig. 3D). We conclude that none of the

2 The mutants are named as follows: single amino acid replacements are named with the letter of the original amino acid, then its position in
the protein and the letter of the new amino acid. The protein in which all the cysteines were replaced is CL. Proteins with single Cys residues
are named CL, followed by the position of the single Cys residue.
TABLE I

| Pretreatment conditions | Mercurial | Wild type | CL-C41 | CL-C95 |
|-------------------------|-----------|-----------|--------|--------|
| No addition             | None      | 540 (100) | 360 (100) | 420 (100) |
| 3 mM IAA                | None      | 545 (101) | 380 (105) | 415 (98) |
| 3 mM MTSEA              | None      | 535 (99)  | 380 (105) | 420 (100) |
| 3 mM NEM                | None      | 510 (94)  | 400 (110) | 420 (100) |
| 1.5 mM PCMB             | None      | 80 (15)   | 120 (33)  | 148 (35) |
| 3 mM IAA                | 1.5 mM PCMB | 68 (12)   | 115 (32)  | 175 (41) |
| 3 mM MTSEA              | 1.5 mM PCMB | 73 (13)   | 115 (32)  | 165 (39) |
| 3 mM NEM                | 1.5 mM PCMB | 76 (14)   | 120 (33)  | 100 (24) |
| No addition             | 1.5 mM PCMBS | ND*      | 176 (48)  | 260 (62) |
| 3 mM IAA                | 1.5 mM PCMBS | ND*      | 152 (42)  | 216 (51) |
| 3 mM MTSEA              | 1.5 mM PCMBS | ND*      | 175 (48)  | 270 (64) |

* ND, not determined.

Fig. 3. None of the Cys residues of EmrE is essential for conferring multiple drug resistance phenotype. E. coli JM109 cells carrying plasmids with various mutants: wild type (□), C39S (●), C41S (○), C95S (▲), CL (■), or mock-transformed vector only (■) were grown at 37°C in LB-Amp medium with different drugs: 400 mM methylviologen (A), 0.4 mM methyl viologen (B), 50 μg/ml acriflavine (C), or none (D). The experiment was repeated twice, and the results were essentially identical.

Cys residues is essential for conferring multiple drug resistance and also EmrE without any Cys (CL) is functional according to this criterion.

To test the level of expression and to quantify the level of activity of each mutant protein, they were extracted with chloroform:methanol and visualized with Coomassie Blue after separation by SDS-polyacrylamide gel electrophoresis. The levels of expression of the various proteins do not differ significantly from the expression of wild type EmrE (data not shown). To test the ability of each mutant to catalyze Δph-driven [14C]methyl viologen accumulation, the proteins were reconstituted into proteoliposomes and transport was measured. The results of such an experiment are shown in Fig. 4. Each of the mutants in which single cysteines were replaced with serine is capable of active accumulation of [14C]methyl viologen. While the wild type protein accumulates about 2100 pmol/min/μg of protein and C41S shows essentially the same rate, C95S accumulates at about 51% (1050 pmol/min/μg of protein) of the above rate and C39S at 32% of the activity (650 pmol/min/μg of protein). In all the single replacements, the steady state levels of accumulation are comparable. The Cys-less protein, in which the three Cys residues were replaced with Ser, displays the lowest activity: only 7–30% of the wild type. The CL protein is particularly poor in its ability to catalyze transport, and a rather high variability in the activity of the CL protein was observed from batch to batch of proteoliposomes. These findings suggest some fluctuation in the stability of this mutant and/or in its ability to reconstitute. Once in proteoliposomes, however, the activity of the CL protein is stable and reproducible.

An important conclusion from these findings is that none of the Cys residues in EmrE is essential for transport. Even though the rates of uptake are significantly modified by the replacements all of them are active and catalyze Δph-dependent accumulation of [14C]methyl viologen.

CL Mutants with Single Cys Display Different Sensitivities to PCMB and PCMBS—Each of the single Cys replacements was sensitive to the inhibitory effects of both mercurials: PCMB and PCMBS (Fig. 5, A and B). The PCMB concentration required to inhibit the rate of transport catalyzed by the wild type protein by 50% (about 1 mM) was very similar to the amount required to inhibit the C95S and C41S mutants. C95S was consistently somewhat more sensitive (0.4 mM IC50). As expected, the CL protein was much less sensitive, but a small and reproducible inhibition was observed which reached a maximum of about 30%, when the concentration of PCMB during the pretreatment was 4.5 mM. PCMB inhibits 50% of the activity of wild type EmrE, C39S, and C95S in the range of...
Cysteine substitutions

![Graph A: Methyl Viologen uptake (percentage of control)]

![Graph B: Methyl Viologen uptake (percentage of control)]

![Graph C: Methyl Viologen uptake (percentage of control)]

![Graph D: Methyl Viologen uptake (percentage of control)]

**Fig. 5.** Different sensitivities to PCMB and PCMBS of mutants with single Cys replacements or CL-EmrE with single cysteines. Proteoliposomes of different EmrE mutants were assayed for initial rate of [14C]methyl viologen uptake after a 7-min preincubation at 22 °C with different concentrations of PCMB (A and C) or PCMBS (B and D). A and B, CL (●), C95S (●●), C41S (●●●), and wild type EmrE (●●●); C and D, CL-C39 (●), CL-C95 (●●), CL-C41 (●●●).

0.3–0.6 mM. However, while it fully inhibited the wild type protein at higher concentrations, it consistently and reproducibly inhibited only 65–70% of the activity of the two mutants. The C41S protein was inhibited only by about 30%. The CL protein was practically unaffected up to 3 mM PCMB. Since in each of the mutant proteins generated above there are still two other Cys residues, to assess which one is the target of action of PCMB, CL proteins with single Cys at their native positions were engineered: CLC39, CLC41, and CLC95. The three above mutants were tested for activity and for their sensitivity to PCMB and PCMBS (Fig. 5, C and D). Each of the single Cys proteins display activities somewhat lower than that shown by the CL protein yet lower than the wild type (see Fig. 4); the range of activities obtained in five different proteoliposome preparations were (in nmol/mg/7 min): 620–800 for CL-C41, 800–1000 for CL-C39, and 720–1000 for CL-C95. The sensitivity to PCMB and PCMBS was tested as described for the other proteoliposome preparations, after exposure to the indicated concentrations and dilution (60-fold) into the assay buffer. CLC41 and CLC95, but not CLC39, are sensitive to the effect of PCMB; the concentration required to inhibit CLC95 is slightly higher (IC50 = 1.5 mM) than that required to inhibit CLC41 (IC50 = 0.5 mM). In both cases, about 80% inhibition is detected at 4.5 mM reagent. CLC39, on the other hand, is only slightly inhibited (about 25%), and this is about the same degree of inhibition observed in CL-EmrE. The general pattern of sensitivity of the various proteins to PCMBS is similar to that described above for PCMB. However, full inhibition is not seen for any of the mutants; it inhibits only 40–50% the CLC95 protein and 60–70% the CL-C41 mutant. Similar to CL-EmrE, the activity of CL-C39 is not influenced at all by PCMBS up to 3 mM in the treatment medium.

From the above studies with the mutants bearing single Cys, we conclude that while residues 41 and 95 are fully exposed to PCMB, they are only partially accessible to PCMBS. Residue 39 is inaccessible to both mercurials. None of the single Cys is exposed to any of the other sulfhydryl reagents tested, since their activity is not influenced by pretreatment with any of them and the reaction with the mercurials is not prevented (Table I).

The partial accessibility to PCMBS in the single Cys replacements as opposed to the full sensitivity of the wild type protein and the reason for the differential exposure to the various sulfhydryl-reactive agents were further probed.

PCMB Is a Substrate of CL-EmrE—As seen above (Fig. 5), PCMB, but not PCMBS, brings about a small but significant inhibition of the activity of the CL and CLC39 protein when the proteoliposomes are pretreated and diluted 60-fold into the assay mixture. This effect of PCMB is due to a reversible and competitive inhibition of the CL protein as shown in Fig. 6A. When PCMB is added directly to the assay medium, a concentration-dependent inhibition is observed with an IC50 of about 150 μM. This concentration dependence is in line with what is observed in Fig. 5 (A and C), where the PCMB was added to the CL EmrE or to CLC39 prior to the reaction (for example at 4.5 mM) and diluted 60-fold to 75 μM; the inhibition observed is about 25%. Since the CL protein has no Cys residues and since the inhibition of CL-mediated transport is reversible, these findings suggest an effect of PCMB independent of its reactivity with sulfhydryls. When this effect is analyzed kinetically, it is seen (Fig. 6A, inset) that the inhibition fits a fully competitive behavior with an apparent Km of 110 μM. The analog that bears a permanent negative charge (PCMBS) has very weak but reproducible inhibitory effect of about 15% at 1 mM and 50% at 3 mM (only part of the range is shown). It is concluded that PCMB behaves as a competitive inhibitor or a substrate of EmrE, while PCMBS is 30-fold less potent.

To test whether PCMB is transported, a pH gradient was generated across the membrane of CL proteoliposomes and they were allowed to accumulate [14C]methyl viologen during 10 min. If no additions were made, uptake continued slowly for the next 5 min and increased by about 20% (Fig. 6B). If, however, PCMB or methyl viologen was added, a rapid decrease of the intraplasomal label was observed. This finding is explained by exchange of the internal label with the excess of added substrates and supports the contention that PCMB is not just an inhibitor but is also transported by CL-EmrE. PCMBS has a very weak effect on the intraplasomal content (data not shown).

Therefore, we suggest that the inhibition by PCMB of the wild type protein and the single cysteine replacements probably reflects the exposure of the Cys residue to the substrate translocation pathway. As described above, none of the residues is accessible to any of the other reagents tested in this work; neither positive nor negative hydrophilic reagents nor small hydrophobic compounds inhibit EmrE. In addition, none of these compounds protects EmrE, CLC41, or CLC95 from the inhibitory effect of PCMB, suggesting that the Cys residues affected by PCMB are in a domain of the protein that is available only to substrates. All the known substrates and inhibitors of EmrE are aromatic compounds bearing a positive charge at given distances of the ring (14). Of the two related mercurials, only PCMB, in which a fraction of the compound is uncharged at given pH values, is a potent inhibitor of the CL mediated transport. PCMBS is only a very weak inhibitor, most likely, because it bears a permanent negative charge.

To further substantiate the hypothesis that PCMB is reacting with Cys residues in the substrate pathway, the effect of a high affinity substrate on the rate of inactivation was tested. The results summarized in Fig. 6C demonstrate that TPP+, a
substrate with an apparent $K_i$ of 8 nM, slowed down the effect of PCMB on the activity of CL-C41 by a factor of about 2-fold. These results are in line with the suggestion that both compounds compete for "sites" on the translocation pathway prior to their reversible reaction of the mercurial with the sulfhydryl on the Cys residue.

The question is then asked: why does PCMBS inhibit even though it is only a very weak substrate? To understand this point, an important difference between the inhibitory effect of both mercurials should be stressed; while both fully inhibit the wild type protein at similar concentrations, their effect on the single Cys protein is quite different. Thus, the activity of CLC95 and CLC41 was only partially inhibited by PCMBS, a compound that cannot cross through the lipid phase and, probably, not through the protein. PCMBS can, therefore, react only with residues exposed to the outside. It is possible then that PCMBS can permeate only part of the substrate pathway and reach exclusively those residues that are close enough to the outside surface. The orientation of the reconstituted EmrE in the proteoliposome is unknown, but, for the sake of simplicity, we assume here that the insertion is random so that about 50% of the molecules are "right side out" and the rest "inside out". In this case, both CLC95 and CLC41 only part of the protein exposes the single Cys to the outside and it is then only partially reachable by PCMBS. The wild type protein, on the other hand, because it is scrambled, exposes in part of the molecules one Cys residue and in the other part the second one. To test this contention, whole cells expressing either CLC95 or CLC41 were treated with PCMB and PCMBS (4.5 mM each). After removal of the compound by centrifugation, EmrE was extracted, reconstituted, and assayed by activity as described under "Experimental Procedures." EmrE from cells expressing either mutant was inhibited practically completely when treated with 4.5 mM PCMB, as expected (Table II). EmrE extracted from cells expressing CLC95 was significantly inhibited (88%) when the cells were challenged with 4.5 mM PCMB. This inhibition is significantly higher than that observed (45–50%) when the CLC95 protein is exposed to the mercurial in proteoliposomes (Fig. 5D). EmrE from cells expressing CLC41, on the other hand, was only marginally inhibited (5–15% in five different experiments), whereas 60–70% inhibition was observed when the reconstituted protein was challenged with the same concentration of the inhibitor (Fig. 5D). To test the premise that this differential effect is due to the fact that residue Cys-41 cannot be reached by PCMBS from the outer face of the membrane, the cells were permeabilized by incubation with 5% chloroform prior to exposure to PCMBS so that the mercurial could readily penetrate. EmrE CLC41 extracted from these cells was now completely inhibited (8% activity, Table II).

**DISCUSSION**

In this work, the role of Cys residues in EmrE has been probed with biochemical tools in combination with a mutagenic approach. Replacement of each of the three Cys residues in EmrE: Cys-39, Cys-41, and Cys-95, had no significant effect on the ability of EmrE to accumulate [14C]methyl viologen in a $\Delta$PH-dependent process. The rates of transport catalyzed by the mutant proteins C39S and C95S are lower than those displayed by the wild type EmrE by about 50%, and the mutant protein devoid of all Cys displays a significantly lower transport capacity. Even though the activity levels of the various mutants vary substantially, they all catalyze a $\Delta$PH-driven...
substrate accumulation and, therefore, it is concluded that none of the Cys in EmrE is essential for catalysis. All the proteins are expressed to comparable levels and confer a similar degree of resistance to three toxicants: methyl viologen, ethidium, and acriflavine. Consequently, it is concluded that only a fraction of the protein activity in the strains expressing EmrE from multicopy plasmids suffices to confer the resistance phenotype. It is therefore impossible to reach quantitative conclusions on the effect of mutations or reagents on EmrE activity solely from observation of resistance in whole cells. EmrE provides a unique experimental system, since it allows for purification of the protein from a culture and functional reconstitution in a very simple, inexpensive, and rapid way. Such a system also allows testing of the effect of various reagents on a highly enriched preparation where no other proteins interfere with the treatment and the driving force is supplied by an artificially imposed pH gradient across the liposome membrane.

Two Cys residues in EmrE have been identified as targets of the inhibitory action of two sulfhydryl-reactive agents: PCMB and PCMBs. These two residues are otherwise unavailable to any of the other reagents tested, whether hydrophobic, hydrophilic, positively or negatively charged, and even though they are smaller and at least as reactive as the mercurials. Because PCMB shows structural similarity to other substrates of EmrE and because it competitively inhibits CL EmrE, it is suggested that Cys-41 and Cys-95 are residues in some area in the transport pathway that is accessible only to the substrate itself. Once in this domain, the mercurials react irreversibly with the sulfhydryl moieties on the protein, a reaction that inactivates EmrE, probably by steric blockage of the passage. PCMB can reach both residues from the outside either because of its ability to cross the passage or because of its high permeability through the lipid bilayer. The fact that PCMB readily exchanges with intraliposomal [14C]methyl viologen implies that it does cross the transport pathway. In addition, the inactivating rate of CLC41 is slowed down in the presence of TPP⁺, a high affinity substrate of EmrE. Cys-39, although close to Cys-41, is located on the other face of the α-helix and is inaccessible to either of the mercurials tested. We conclude that Cys-39 is either buried in the polypeptide or facing the lipid.

The effect of PCMBs differs from that of PCMB in several aspects. From its inhibition of transport catalyzed by CL-EmrE (IC₅₀ = 3 mM), PCMBs seems to be interacting only very weakly with the protein. That it is interacting in a domain closer to the external part of the translocation pathway, we conclude from the asymmetric irreversible inactivation. It is likely that the negative charge in the molecule prevents it from going completely through, yet it can interact with domains unreachable to the other reagents tested. Its asymmetric effect is in line with the hypothesis that the topology of the reconstituted protein in the proteoliposome is mixed and practically random yielding about 30–50% in the configuration similar to that observed in whole cells (right side out, Cys-95 exposed) and the other 50–70% in an inverse configuration (inside out, Cys-41 exposed). Taken together the results suggest that Cys-41 can be reached by PCMBs only from the cytoplasmic face of the membrane. Cys-95 is fully accessible in intact cells, but since the accessibility in proteoliposomes is only partial, we conclude that it is accessible exclusively from the outside face of the cell.

The reactivity of Cys to small sulfhydryl-reactive agents has been previously used to map the residues lining the channel pathway in several cases (16–18). Cys residues were systematically engineered in domains previously suspected to line the channel, and their ability to change the conductance properties were recorded. In these cases the selectivity is determined by the small size and by the charge of the probing reagent, but, aside from that, most of the channel is freely accessible. In other transporters, proximity to the substrate path is inferred from protection by substrates against inactivation. In ion-coupled transporters, an extensively studied case is that of a Cys residue (Cys-148) in the Lac permease, a H⁺/β-galactoside symporter from E. coli. Substrate protection experiments (29) and biophysical studies (30) suggest that Cys-148 is located in the vicinity of the substrate binding site.

In the case of EmrE, a unique property of the domain identified is its inaccessibility to compounds other than the substrate, in our case PCMB. This domain is lined by Cys-41 and Cys-95. In addition, we conclude that these residues are asymmetric in their location with respect to the plane of the membrane; Cys-95 is closer to the outside face of the membrane, and Cys-41 is closer to the inner face. Based on the findings presented, we suggest the existence in the translocation pathway in EmrE of a domain accessible only to substrates, a "selectivity filter" to which access is limited. It will be interesting to determine the size of this domain relative to subdomains in which access is unlimited. In this respect, in wild type Smr, a protein homologous to EmrE from Staphylococcus aureus, there is a single Cys in a position equivalent to position 43 in EmrE. This Cys is accessible to NEM and the process is slowed down by substrates (13). The findings described in this manuscript provide us with a tool to dissect the different domains in the substrate path in EmrE by cysteine mutagenesis.

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