EmrE, a Multidrug Transporter from Escherichia coli, Transports Monovalent and Divalent Substrates with the Same Stoichiometry*

Dvir Rotem‡ and Shimon Schuldiner§

From the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

Multidrug transporters recognize and transport substrates with apparently little common structural features. At times these substrates are neutral, negatively, or positively charged, and only limited information is available as to how these proteins deal with the energetic consequences of transport of substrates with different charges. Multidrug transporters and drug-specific efflux systems are responsible for clinically significant resistance to chemotherapeutic agents in pathogenic bacteria, fungi, parasites, and human cancer cells. Understanding how these efflux systems handle different substrates may also have practical implications in the development of strategies to overcome the resistance mechanisms mediated by these proteins.

Here, we compare transport of monovalent and divalent substrates by EmrE, a multidrug transporter from Escherichia coli, in intact cells and in proteoliposomes reconstituted with the purified protein. The results demonstrated that whereas the transport of monovalent substrates involves charge movement (i.e. electrogenic), the transport of divalent substrate does not (i.e. electroneutral). Together with previous results, these findings suggest that an EmrE dimer exchanges two protons per substrate molecule during each transport cycle. In intact cells, under conditions where the only driving force is the electrical potential, EmrE confers resistance to monovalent substrates but not to divalent ones. In the presence of proton gradients, resistance to both types of substrates is detected. The finding that under some conditions EmrE does not remove certain types of drugs points out the importance of an in-depth understanding of mechanisms of action of multidrug transporters to devise strategies for coping with the problem of multidrug resistance.

Multidrug transporters (MDTs)\(^3\) and drug-specific efflux systems are responsible for clinically significant resistance to chemotherapeutic agents in pathogenic bacteria, fungi, parasites, and human cancer cells (1–3). Phylogenetic studies show that these efflux systems are associated with five superfamilies of transporters (4). One of these includes a family of small multidrug resistance (SMR) conferring proteins. The SMR family consists of small hydrophobic proteins of 100 amino acid residues with four transmembrane \(\alpha\)-helical spanners (5–7). These proteins remove cationic drugs from the cytoplasm using a drug/H\(^+\) antiport mechanism (5–7).

Genes coding for SMR proteins have been identified in many eubacteria and in some Archaea (8, 9). The most extensively characterized SMR protein is EmrE, from Escherichia coli. The protein has been characterized, purified, and reconstituted in a functional form (10). High-affinity substrate binding has been established as a reliable and sensitive assay for activity of the detergent-solubilized transporter (11–13).

Glu-14, the only membrane-embedded charged residue, is highly conserved in the SMR family (8). This residue has an unusually high \(pK_a\) and is an essential part of the binding domain shared by substrates and protons (11–13). The occupancy of the binding domain is mutually exclusive, and as such this provides the molecular basis for coupling of substrate and proton fluxes. Direct measurements of substrate-induced release of protons in a detergent-solubilized EmrE shows the stoichiometry of the release is almost 1 proton per monomer. The findings demonstrate that the only residue involved in proton release is Glu-14 and that all the Glu-14 residues in the EmrE functional oligomer participate in proton release (14).

MDTs transport substrates with apparently little common structural features. At times these substrates are neutral, negatively, or positively charged (15–18). This poses intriguing mechanistic challenges such as binding of substrates with different charges to a common binding domain. Some clues for the molecular basis of multidrug recognition are emerging from the structural studies of transcription factors that regulate expression of MDTs (19). Only limited information is available as to how the MDTs deal with transport of substrates with different charges. The gradient that ion-coupled transporters can generate depends on the number of ion molecules transported per substrate molecule (stoichiometry) and on the charge of the substrate. In the case of MdA, an E. coli multidrug transporter of the major facilitator superfamily, it was shown that it exchanges neutral compounds as chloramphenicol and thiamphenicol and monovalent cationic substrates with the same stoichiometry, and as a result the driving forces for both types of substrates are different (20).

In this study, we compare transport of monovalent and divalent substrates by EmrE in intact cells and in proteoliposomes reconstituted with the purified protein. In intact cells, under conditions where the only driving force is the electrical potential, EmrE confers resistance to monovalent substrates but not to divalent ones. In the presence of a proton gradient, resistance to both types of substrates is detected. In proteoliposomes reconstituted with the purified protein. In intact cells, under conditions where the only driving force is the electrical potential, EmrE confers resistance to monovalent substrates but not to divalent ones. In the presence of a proton gradient, resistance to both types of substrates is detected. In proteoliposomes reconstituted with the purified protein. In intact cells, under conditions where the only driving force is the electrical potential, EmrE confers resistance to monovalent substrates but not to divalent ones. In the presence of a proton gradient, resistance to both types of substrates is detected.
posomes reconstituted with purified EmrE only proton grad-
ients were able to drive transport of both divalent and mono-
valent substrates, whereas the electrical potential drove
transport of monovalent substrates. The results demonstrated
that whereas the transport only of monovalent substrates in-
volves charge movement (i.e., electrogenic), the transport of
divalent substrate does not (i.e., electroneutral). Together with
previous results, these findings suggest that an EmrE dimer
exchanges two protons per substrate molecule during each
transport cycle. The findings that under some conditions EmrE
is ineffective in removing certain types of drugs imply that an
in-depth study of mechanisms of action of MDTs may help
devising strategies for coping with the problem of multidrug
resistance.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** E. coli JM109 (21) and TA15
strains (22) are used throughout this work. The plasmids used are
pT7–7 (23) derivatives for wild type with (EmrE-His (11)) or without
(10) His-tag.

**Resistance to Toxic Compounds—** Resistance to toxic compounds
methyl viologen (MV²⁺), ethidium, or acriflavine was tested by meas-
uring optical density of 0.3-ml cultures after overnight growth of JM109
cells bearing plasmids pT7–7 with or without the EmrE gene. Growth
was at 37 °C in a 1.2-ml-square storage plate (96 wells, Abgene, Surrey,
UK). LB medium containing 50 μg/ml ampicillin and 30 mM Bis-Tris
propane was titrated to the indicated pH with HCl. Bis-Tris propane
was added to maintain a constant pH during growth as described
previously (24).

**Overexpression, Purification, and Reconstitution of EmrE-His—**
Overexpression and purification of EmrE was performed essentially as
described previously (14). For reconstitution, purified EmrE-His was
incubated with nickel-nitrilotriacetic acid beads (Qiagen GmbH,
Hilden, Germany) for 1 h at 4 °C. The beads were washed once with
buffer containing 150 mM NaCl, 15 mM Tris–Cl, pH 7.5 (sodium buffer)
with 0.08% n-dodecyl-β-D-maltoside (Glycon Biochemicals, Lucken-
walde, Germany), 15 mM β-mercaptoethanol, and 3 × in sodium buffer
containing 1% n-octyl-β-D-glucopyranoside (Glycon Biochemicals, Luck-
kenwalde, Germany). EmrE-His was eluted from the beads with sodium
buffer containing 1% n-octyl-β-D-glucopyranoside and 200 mM imidaz-
ole. The supernatant was mixed with a solution containing 25 mg/ml
E. coli phospholipids (Avanti, Inc., Alabaster, AL), 150 mM NaCl, 15 mM
Tris–Cl, pH 7.5, 1% n-octyl-β-D-glucopyranoside. The amount of protein
mixed with the phospholipids is indicated in the specific experiments.
To generate ΔpH, after sonication the mixture was diluted 25-fold into
NH₄ buffer containing 190 mM NH₄Cl, 15 mM Tris–Cl, pH 7.5, and 1 mM
dithiothreitol, incubated for 20 min at 25 °C, and then centrifuged at
24000g for 10 min. Proteoliposomes were resuspended in NH₄ buffer,
flushed in liquid and air, and kept at −70 °C.

To generate Δψ, the proteoliposomes were formed by dilution into 70
mM sodium thiocyanate, 120 mM NaCl, 20 mM Tris–Tricine buffer, pH 7.5
and 1 mM dithiothreitol (thiocyanate buffer). They were then cen-
trifuged, resuspended and frozen as above. Before the assay, the
proteoliposomes suspension was thawed and sonicated in a bath-type son-
icator for a few seconds until clear.

**ΔpH-driven Methyl Viologen Uptake Assay—** Uptake of [¹⁴C]methyl
viologen into proteoliposomes was assayed essentially as described in
Yerushalmi et al. (10). 3 μl of the NH₄ containing proteoliposomes was
diluted into 200 μl of an ammonium-free solution. The latter contained
20 μM [¹⁴C]methyl viologen (32 nCi/assay), 140 mM KCl, 10 mM Tricine,
10 mM Tris, and 5 mM MgCl₂ (final pH 8.5) (KCl buffer). At given times
the reaction was stopped by dilution with 2 ml of the same ice-cold
solution. The samples were filtered through Millipore filters (0.22 μm)
and washed with an additional 2 ml of solution. The radioactivity on the
samples was measured by liquid scintillation. In each experiment the
values obtained in a control reaction with 15 μm nigericin were sub-
tracted from all experimental points. This background was no more than
1% of the maximal experimental values. The experiment was performed in
duplicates. Each experiment was performed at least twice.

**ΔpH-driven Tetraphenylphosphonium (TPP⁺) Uptake Assay—** 3 μl
of NH₄ containing proteoliposomes were diluted into 200 μl of KCl buffer
that contained 10 mM [⁴¹⁷]TPP⁺ (60 nCi/assay). Because of the relatively
high nonspecific binding of [⁴¹⁷]TPP⁺ to the filters, the reaction was
slightly modified so that it was stopped by centrifugation (452 × g, 1
min) through columns (2-ml disposable syringes) containing Sephadex
G-50 fine, preswollen in KCl buffer and packed by centrifugation (113 ×
g, 1 min). The radioactivity was measured by liquid scintillation. In
each experiment, a control reaction was carried out where no gradient
was generated (dilution into ammonium buffer). The values obtained in
such a reaction were subtracted from all experimental points. The
reactions were carried out in duplicates. Each experiment was per-
formed at least twice.

For examining the effect of N,N′-dicyclohexylcarbodiimide (DCCD)
on [⁴¹⁷]TPP⁺ uptake, proteoliposomes were incubated with 0.3 mM
DCCD for 30 min before the uptake assay. Drug gradients were calcu-
lated using intraliposomal volumes determined in a separate experi-
ment as follows: proteoliposomes were sonicated in the presence of 2 mM
[¹⁴C]methyl viologen (32.3 μCi/μmol). A sample (3 μl) was diluted with 2
ml of the same ice-cold solution used in the transport experiments,
filtered through Millipore filters (0.22 μm), and washed with an addi-
tional 2 ml of solution. From the radioactivity associated with the
proteoliposomes an internal volume of 0.2 μl per sample was calculated.
To prevent significant leakage this manipulation was performed
with proteoliposomes reconstituted with EmrE E14C, an inactive
mutant (10).

**ΔpH-driven Uptake Assays—** Δψ driven uptake assays of MV²⁺
and TPP⁺ were performed as the ΔpH-driven uptake assays except that
the inner and outer solutions differed in composition, 3 μl of proteolipo-
somes formed in thioxonate buffer were diluted into 200 μl of a solu-
tion containing 10 mM [⁴¹⁷]TPP⁺ or 41 μl [¹⁴C]MV²⁺, 190 mM KCl, and
20 mM Tris–Tricine buffer, pH 7.5. As a control the proteoliposomes
were diluted into a buffer of composition identical to the one in their
interior. Results were essentially identical when the membrane potent-
ial was generated by a valinomycin-induced K⁺ diffusion potential in
a medium with no chloride as described in Ref. 20.

**RESULTS**

**The Resistance That EmrE Confers against Monovalent and
Divalent Substrates Is Affected Differently by Changes in the
External pH—** E. coli cells that overexpress EmrE display re-
sistance to a wide variety of toxic lipophilic cations, monoval-
ents such as ethidium and acriflavine, and divalents such as
MV²⁺ (10). The growth of E. coli cells carrying plasmids with or
without the emrE gene in LB medium containing monovalent
or divalent lipophilic cations was compared under various pH
conditions (Fig. 1). In the absence of drugs, cells harboring
either plasmid grew equally well in the pH range of 7.0–8.4
(data not shown). As expected when the monovalent substrates
125 μM ethidium or 62 μM acriflavine were added to the me-
dium, only cells expressing EmrE were able to grow. At these
drug concentrations growth was practically identical in the pH
range tested. In contrast, the resistance that EmrE confers
against 62 μM of the divalent substrate MV²⁺ is drastically
reduced as the pH increases, and it is abolished above pH 7.6.

To further explore the nature of this different sensitivity to
pH, a more detailed study was carried out and the IC₅₀ values of the
three compounds were determined at the various pH values.
The findings are illustrated for two pH values in each case
(Fig. 2, A–C) and summarized in Fig. 2, right panels. Ethidium
and acriflavine markedly inhibited growth at pH 7.0 and pH
8.4 with a slightly higher potency (about 5-fold) at the more
alkaline pH (Fig. 2, A and B, gray lines). At the two pH values,
EmrE significantly protected against the toxic effects of both
compounds (black lines). In the case of MV²⁺, the difference
between the two pH values is more striking. MV²⁺ was 15
times more potent at pH 8.4 than at pH 7.0 (Fig. 2C, gray lines).
The protection conferred by EmrE at pH 8.4 was only slightly
evident at the low concentration (black lines). Moreover, at this
pH, EmrE induces a reproducible and significant sensitization
at the higher concentrations.
EmrE Catalyzes Uptake of TPP⁺ into Proteoliposomes—EmrE catalyzes ΔpH-driven uptake of MV²⁺ and ethidium into proteoliposomes (10). Transport of MV²⁺ can be measured rapidly and quantitatively using radiolabeled substrate (10). Transport of ethidium is followed using an assay based on changes of fluorescence of ethidium upon uptake to proteoliposomes loaded with nucleic acids. This is a convenient assay but only qualitative. Therefore it was necessary to introduce a new assay and we chose to study transport of TPP⁺, a compound that binds to detergent-solubilized EmrE with nanomolar affinity (11). We chose TPP⁺ because of its high affinity and because of the knowledge we have accumulated on its interaction with EmrE (6, 7). In these experiments, the driving force was a pH gradient generated by the ammonium diffusion gradient obtained upon dilution of the proteoliposomes prepared in NH₄Cl medium into media in which the ammonium was replaced by KCl (10). A time-dependent accumulation of [³H]TPP⁺ was observed in proteoliposomes reconstituted with EmrE (Fig. 3A, black squares). When the pH gradient was abolished by dilution into a medium identical in composition to that inside the proteoliposome, a small rapid association of TPP⁺ to the proteoliposomes was observed (Fig. 3A, white squares). As shown in Fig. 3B, other substrates of EmrE, like MV²⁺, ethidium, and acriflavin inhibit the [³H]TPP⁺ uptake in a concentration range expected from their kinetic properties.

In Fig. 2, right panels, we can see the systematic decrease of IC₅₀ values for all of the substrates tested at the high pH values. However, whereas the drop in IC₅₀ for ethidium and acriflavin in the presence of EmrE was about 8- and 4-fold, respectively, in its absence there was also an increased toxicity (Fig. 2C). The internal pH of E. coli cells is kept in the range 7.0–7.9 (25). As a result, as external pH is raised, IC₅₀ drops and even becomes inverted (inside acid, outside alkaline) drops and even becomes inverted (25). This raises the hypothesis that EmrE can only utilize ΔpH to extrude MV²⁺ from the cells, suggesting the possibility that transport is electroneutral. Above pH 7.8–8.0, only compounds that are electrogenically transported out of the cell can be removed. In contrast, at high pH, without a significant driving force EmrE may facilitate the entry of compounds that are transported electroneutrally. This can explain the higher sensitivity to MV²⁺ of cells that express EmrE at high pH (Fig. 2C).

Therefore, a detailed study of the effect of the components of the proton electrochemical gradient on transport of monovalent and divalent substrates was carried out.

Drives EmrE-mediated Uptake of TPP⁺ but Not of MV²⁺—Depending on the proton/substrate stoichiometry of the reaction and the charge on the substrate, exchange of protons with a substrate by antiporters may result in a net movement of electric charges (electrogenic transport) or no net movement of charges (electroneutral transport). Electrogenic transport will be driven by both components (ΔΨ and ΔpH) of the proton electrochemical gradient (Δfₑ⁻), but when transport is electroneutral, ΔpH is the primary driving force (27, 28). To determine whether EmrE can utilize ΔΨ as the driving force for uptake of substrates, we used proteoliposomes where ΔΨ (positive inside) was generated by imposing a gradient of SCN⁻, a permeant anion (SCN⁻ₐₙ > SCN⁻ₐₒᵤₜ). The results shown in Fig. 5 demonstrate EmrE-catalyzed uptake of the monovalent cat-

![Image](https://example.com/image.png)

**Fig. 1. Resistance to Toxic Compounds at Different pH.** Resistance to toxic compounds acriflavin (62 μM) (A), ethidium (125 μM) (B), or methyl viologen (62 μM) (C) was tested after overnight growth of JM109 cells bearing plasmids pT7-7 with (black squares) or without (white squares) the EmrE gene. Growth was for 14–16 h at 37 °C in LB medium containing 50 μg/ml ampicillin and 30 mM Bis-Tris propane titrated to the indicated pH with HCl.

In Fig. 2, right panels, we can see the systematic decrease of IC₅₀ values for all of the substrates tested at the high pH values. However, whereas the drop in IC₅₀ for ethidium and acriflavin in the presence of EmrE was about 8- and 4-fold, respectively, in its absence there was also an increased toxicity (Fig. 2C). The internal pH of E. coli cells is kept in the range 7.0–7.9 (25). As a result, as external pH is raised, IC₅₀ drops and even becomes inverted (inside acid, outside alkaline) drops and even becomes inverted (25). This raises the hypothesis that EmrE can only utilize ΔpH to extrude MV²⁺ from the cells, suggesting the possibility that transport is electroneutral. Above pH 7.8–8.0, only compounds that are electrogenically transported out of the cell can be removed. In contrast, at high pH, without a significant driving force EmrE may facilitate the entry of compounds that are transported electroneutrally. This can explain the higher sensitivity to MV²⁺ of cells that express EmrE at high pH (Fig. 2C).
Fig. 2. Determination of IC₅₀ values of monovalent and divalent substrates. Growth of cells bearing plasmids pT7–7 with (black lines, squares) or without (gray lines, triangles) the EmrE gene at different pH conditions at different concentrations of acriflavine (A), ethidium (B), or methyl viologen (C) was tested. Growth was at 37 °C in LB medium containing 50 μg/ml ampicillin, 30 mM Bis-Tris propane titrated to the indicated pH with HCl, and different concentrations of the drugs. The experiment was repeated four times. The results of experiments at pH 7.0 (solid lines, black symbols) and pH 8.4 (dashed lines, white symbols) are presented in the left panel for illustration. Each curve is result of a fit obtained using Origin 7.0 software (OriginLab, Northampton, MA). The IC₅₀ values from all the pH values tested are summarized in the right panel. At the pH values above 7.8 and indicated with a * there is a slight EmrE-dependent resistance at low concentrations of MV²⁺, and EmrE-dependent sensitization at high concentrations are as shown for pH 8.4 in the left panel. IC₅₀ values were calculated with Origin 7.0 software.

ion TPP⁺ into proteoliposomes when ΔΨ (positive inside) is generated (filled squares). TPP⁺ uptake was abolished with DCCD, a carbodiimide that inhibits TPP⁺ binding to the protein (data not shown). The TPP⁺ concentration gradient generated by EmrE about 60-fold is almost identical to the predicted accumulation expected from the SCN⁻ gradient generated by dilution (66-fold) if one charge is moving per transport cycle. TPP⁺ has been shown to equilibrate across membranes in an unmediated mode in response to electrical potentials and has been used to estimate their magnitudes (29). Here, TPP⁺ accumulates despite the fact that the potential is positive inside, suggesting that under these conditions the EmrE-mediated flux is much faster than the unmediated one. The above findings suggest that, by definition, TPP⁺ transport is electroneutral, and the stoichiometry of the exchange reaction H⁺/TPP⁺ is therefore higher than 1.

On the other hand, under the same conditions, EmrE does not catalyze MV²⁺ uptake (Fig. 5, white squares). These results indicate that unlike with the monovalent substrate TPP⁺, ΔΨ cannot drive transport of the divalent substrate MV²⁺, whereas ΔpH drives transport of both substrates. Again, by definition we conclude that MV²⁺ transport is electroneutral, and therefore the stoichiometry of the exchange reaction H⁺/MV²⁺ is 2.

The Protonophore CCCP Affects TPP⁺ but Not MV²⁺ Uptake into Proteoliposomes—Another way to determine whether TPP⁺ transport by EmrE is electroneutral and MV²⁺ transport is electroneutral is by manipulation of gradients with ionophores. The equilibrium equation for a substrate-proton exchange reaction is given by Equation 1,

\[ \frac{RT}{F} \ln \frac{S_i}{S_o} = n_H \frac{RT}{F} \ln \frac{H_i}{H_o} + \Delta \phi (n_H Z_H - Z_s) \]  
(Eq. 1)

where \( RT \) and \( F \) are physical constants, \( S \) and \( H \) denote the substrate and proton concentration in the inner (i) and outer (o) compartment, \( n_H \) stands for the stoichiometry H⁺/substrate, and \( Z \) is the charge of the substrate (s) or the proton (H).

Thus, for a divalent substrate, transport will be electroneutral when \( n_H = 2 \). In other words, under these conditions the membrane potential will not affect transport. When a pH gradient is generated (for example by an ammonium diffusion gradient) the addition of a protonophore such as carbonylcyanide m-chlorophenylhydrazone (CCCP) will generate a proton diffusion membrane potential equal to ΔpH but of opposite magnitude. The uptake of MV²⁺ into EmrE proteoliposomes was not affected by the addition of CCCP (Fig. 6A). Allowing the downhill movement of H⁺ by the addition of the K⁺ ionophore valinomycin, together with CCCP, discharged the ΔpH and inhibited MV²⁺ uptake (Fig. 6A). Similarly, the addition of the ionophore nigericin, which exchanges H⁺ and K⁺ ions, dissipates the pH gradient and inhibits MV²⁺ uptake.
As shown by this equation, when the transport is electrogenic, transport will depend on $\Delta \mu^+_{\text{H}^+}$ with a bias on each component depending on the stoichiometry. Thus when $n_{\text{H}^+} = 2$, the equilibrium equation for a substrate-proton exchange reaction is given by Equation 2.

$$\frac{RT}{F} \ln \frac{S_i}{S_0} = 2 \left( \frac{RT}{F} \ln \frac{H_i}{H_0} \right) + \Delta \phi$$

(Eq. 2)

The results in Fig. 6B show that the accumulation of $\text{TPP}^+$ into EmrE proteoliposomes was significantly albeit not fully reduced upon the addition of the protonophore CCCP. $\text{TPP}^+$ uptake is inhibited when a combination of CCCP and valinomycin (Fig. 6B) or the ionophore nigericin (data not shown), which collapse $\Delta \mu^+_{\text{H}^+}$ under these conditions, are added. That CCCP does not fully inhibit $\text{TPP}^+$ uptake is explained by the fact that, as shown previously, $\text{TPP}^+$ accumulates inside liposomes as dictated by the membrane potential (inside negative) (29). This is illustrated in Fig. 6C where liposomes that do not contain protein cannot accumulate $\text{TPP}^+$ when a pH gradient is generated. However, after the addition of CCCP (diffusion potential negative inside) $\text{TPP}^+$ accumulates to levels similar to those observed in EmrE containing proteoliposomes.

**DISCUSSION**

EmrE transports a wide variety of aromatic cations. A comparison between the uptake of radiolabeled monovalent and divalent substrates into EmrE proteoliposomes was conducted under conditions where the driving forces were either a proton gradient or a transmembrane electrical potential. The results demonstrate that both forces drive the uptake of the monovalent substrate $\text{TPP}^+$, but only the proton gradient can drive uptake of the divalent substrate $\text{MV}^{2+}$. We conclude that the transport of monovalent substrates is electrogenic whereas the transport of the divalent ones is electroneutral. The difference in the transport modes is most likely because of the fact that the transporter uses the same proton/substrate stoichiometry with all the substrates. Because the valence of the substrates...
differs, different amounts of charge are translocated in one transport cycle. The findings imply that EmrE exchanges two protons per substrate.

High affinity ligand binding studies, functional complementation, and negative dominance studies all suggested that EmrE functions as an oligomer, but the size of the functional oligomer was not definitely established (11, 30, 31). Structural evidence from two-dimensional crystals led to the proposal that the minimal functional unit of EmrE was likely to be a dimer (32). This was confirmed by the determination of the three-dimensional structure of EmrE with TPP$^+$ bound at 7.5 Å resolution by electron cryomicroscopy of two-dimensional crystals (33). The minimal structural unit is an asymmetric homodimer composed of eight transmembrane α-helices, i.e. four helices from each monomer, with density for TPP$^+$ in a binding chamber formed from six of the 8 α-helices, confirming the suggestion that TPP$^+$ binds near the center of the dimer (33). However, the two-dimensional crystals do not rule out the existence of higher order oligomers. In fact, co-expression of two plasmids in a cell free system allowed demonstration of functional complementation, and pull-down experiments confirmed that the basic functional unit is the dimer (34). An additional weaker interaction between dimers detected using cross-linking implies the existence of a dimer of dimers (34). In addition, in a model based on three-dimensional crystal diffraction to 3.8 Å, the protein is suggested to be a dimer of dimers (35).

The evidence presented in this study shows that the stoichiometry of the exchange reaction is two protons per substrate. Previous results showed that substrate binding to detergent-solubilized EmrE induces the release of almost one proton per EmrE monomer (14). Taking these two results together, the simplest conclusion is that the functional transport unit is the dimer. In the model mentioned above (35), the tetramer is the basic unit implying that substrate binding to EmrE would induce the release of 0.5 proton per EmrE monomer, a suggestion that is not borne out by experimental evidence (35).

Detergent-solubilized EmrE binds TPP$^+$ with high affinity, and the study of this reaction has provided important information about the mechanism of catalysis by EmrE (11, 12). The measurements of ligand binding in detergent were validated by measurements of the pH profile of MV$^{2+}$ transport in proteoliposomes reconstituted with purified EmrE (36). The finding that the protein binds TPP$^+$ also when it is embedded in the phospholipid bilayer and that TPP$^+$ is not only a ligand but also a bona fide substrate of EmrE provides a more direct substantiation of the binding measurements with detergent-solubilized protein. Transport is easily detected under the conditions used despite the fact that TPP$^+$ is a lipophilic cation with a relatively high permeability through biological membranes. Up to now, we have not been able to show a significant resistance to TPP$^+$ in intact cells expressing EmrE (17). The direct demonstration of transport in this work and the well known fact that TPP$^+$ is a permeant cation that equilibrates with the membrane potential suggest that the lack of resistance is caused by the fact that the efflux rates catalyzed by EmrE in intact cells (with very low expression) were in the same order of magnitude of the passive uptake of TPP$^+$.

We demonstrated here that the sensitivity of E. coli cells that express EmrE to monovalent drugs ethidium and acriflavine is much less affected by the rise in the external pH compared with the sensitivity to the divalent drug MV$^{2+}$. In growing E. coli cells $\Delta\mu_{H^+}$ remains relatively constant at external pH values

\[ \text{FIG. 6. The effect of ionophores on TPP$^+$ and MV$^{2+}$ uptake.} \]

A, ammonium-loaded EmrE proteoliposomes (3 μl) were diluted into ammonium-free medium to generate a pH gradient. In addition the medium contained (A) $[^{14}C]\text{MV}^{2+}$ or (B) $[^{3}H]\text{TPP}^+$. Ionophores were added where indicated to the following concentrations: CCCP, 5 μM; valinomycin, 100 nM; nigericin, 15 μM. In C, the liposomes used are without protein. In A the reaction was terminated after 5 min, and in B and C the reaction was terminated after 1 min as described under “Experimental Procedures.”

\[ \text{48792 Transport of Monovalent and Divalent Substrates by EmrE} \]
ranging from 5.0—8.0, whereas the electrical ($\Delta V$) and chemical ($\Delta pH$) components interconvert. Because internal pH is kept constant at 7.6 as external pH is raised, $\Delta pH$ (inside alkaline) drops and even becomes inverted (inside acid) above pH 7.6, whereas $\Delta V$ (inside negative) increases (25, 37). We conclude that the difference in the sensitivity results from the fact that only the electrogenic exchange of the monovalent substrates utilizes the membrane potential, whereas there is no driving force for the removal of divalent substrates at the high pH. Even though the $\Delta pH$ remains relatively constant through the pH range tested, the sensitivity to the monovalent substrates increases at alkaline pH. As discussed above (Equation 2), this is because of the fact that when the stoichiometry $H^+/substrate$ is higher than 1, a decrease in $\Delta pH$ has a stronger effect on the substrate gradient that can be generated than the equivalent increase of $\Delta V$. Interestingly, the sensitivity of the cells that do not express EmrE increases at the alkaline pH values, again in a more pronounced form for the divalent $MV^{2+}$. This may be caused by increased entry of the compounds because of the higher membrane potential, increased toxicity, or a reduced ability of the “housekeeping” multidrug transporters to remove the cations. The latter can be due again to the fact that also other transporters may transport divalent cations electrogenically and monovalent electroneutrally. Several other antibiotics and toxins seem to be more effective at alkaline pH (20, 38, 39). The ability to transport substrates with different valence has been reported for other bacterial secondary multidrug transporters; for example, QacA from *Staphylococcus aureus* confers resistance against a wide range of mono- and divalent lipophilic cations (16), and *E. coli* MdfA confers resistance against monovalent lipophilic cations and neutral compounds (15). As already mentioned, MdfA exchanges neutral compounds as chloramphenicol and thiampenicol in an electrogenic mode, whereas it exchanges monovalent cationic substrates in an electroneutral mode (20).

These findings suggest a general property of multidrug transporters that recognize substrates with different valences and transport them in different modes. This could provide a novel strategy to overcome some of the drug resistance infections in the clinic because we could in theory engineer conditions where the MDTs are not efficient (for example whenever it is possible to raise the local pH of the environment) or develop new compounds that, under physiological conditions, will be poor substrates of the MDTs.

Acknowledgments—We thank Samanta Jamzon for performing some of the experiments and Misha Soskine for providing purified EmrE for the reconstitution.

REFERENCES