EmrE, a member of the small multidrug transporters superfamily, extrudes positively charged, hydrophobic compounds out of *E. coli* cytoplasm in exchange for inward movement of protons down their electrochemical gradient. Although its transport mechanism has been thoroughly characterized, the structural basis of energy coupling and the conformational cycle mediating transport are yet to be elucidated. In this paper, EmrE structure in liposomes and the substrate-induced conformational changes were investigated by systematic spin labeling and electron paramagnetic resonance (EPR) analysis. Spin label mobilities and accessibilities describe a highly dynamic ligand-free (apo) conformation. Dipolar coupling between spin labels across the dimer reveals at least two spin label populations arising from different packing interfaces of the EmrE dimer. One population is consistent with antiparallel arrangement of the monomers although the EPR parameters suggest deviations from the crystal structure of substrate-bound EmrE. Resolving these discrepancies requires an unusual disposition of TM3 relative to the membrane-water interface and a kink in its backbone that enables bending of its C-terminal part. Binding of the substrate tetraphenyl phosphonium (TPP⁺) changes the environment of spin labels and their proximity in three transmembrane helices. The underlying conformational transition involves repacking of TM1, tilting of TM2, and changes in the backbone configurations of TM3 and the adjacent loop connecting it to TM4. A dynamic apo conformation is necessary for the polyspecificity of EmrE allowing the binding of structurally diverse substrates. The flexibility of TM3 may play a critical role in movement of substrates across the membrane.

One mechanism of multidrug resistance involves the active extrusion of toxic molecules out of the cell by dedicated membrane transporters. In prokaryotes, five superfamilies of transporters handle vectorial drug traffic moving energetically uphill against substrate concentration gradients (1). The thermodynamics of the problem are rendered favorable through coupling of substrate movement to the direct use of ATP¹
energy or the discharge of electrochemical ion gradients. Small multidrug resistance (SMR) transporters are the smallest bacterial transporters with four predicted transmembrane α-helices and no significant extramembrane domain (2,3). They function as dimers coupling translocation of positively charged hydrophobic substrates out of the cell to the inward movement of protons.

Much of the current mechanistic understanding of SMR transporters has emerged from seminal studies in the laboratory of Shimon Schuldiner defining fundamental steps in the transport cycle of \textit{E. coli} EmrE (4-7). Protons and substrates share a common binding site organized around the absolutely conserved, membrane-embedded, glutamate 14 (E14) in transmembrane helix 1 (TM1) (8). Binding of positively charged hydrophobic substrates is coordinated by E14 side chain and stabilized by interactions with aromatic residues in TM segments 1-3 (9-11). Proton release at the cytoplasmic side, facilitated by the perturbed pKa of E14, is concomitant with substrate binding (12). Mutually exclusive occupancy of the binding site by protons and substrates provides the basis for coupling of the two fluxes (13,14).

The structural framework that mediates vectorial transport continues to be controversial. Specifically, the orientation of the two monomers in the dimer has emerged as a focal point of contention. EmrE structures, determined from 2 and 3 dimensional crystals (15-17), provide compelling evidence supporting antiparallel orientation of the monomers. In addition, the sequence determinants of membrane topology, the arginine and lysine contents of EmrE loops (i.e. the K+R bias), do not favor a unique orientation in the membrane implying that EmrE could insert with dual topology (18). However, experiments designed to verify or alter the relative orientation of the monomers were inconclusive (19).

In contrast, a body of biochemical data supports a parallel orientation of the dimer. These include the design and construction of functional EmrE chimeras where monomers are linked by short polar loops not favored energetically to cross the bilayer (20). Furthermore, residues predicted to be on opposite sides of the membrane in the antiparallel dimer model can be cross-linked without significant perturbation of transport (21). Finally, limited structural constraints derived from EPR analysis of spin labeled EmrE in TM3 were interpreted as consistent with a parallel orientation (19).

Contributing to the structure mechanism divide is the absence of direct analysis of the structure and conformational dynamics of EmrE in lipid bilayers in various transport intermediates. Specifically, the structural changes induced by substrate/proton binding have not been described as both available structures were obtained in the presence of the substrate tetraphenyl phosphonium (TPP⁺). In this paper, we report results from a systematic analysis of EmrE structure in the (substrate-free) apo and TPP⁺-bound conformations in liposomes. For this purpose, spin label probes were introduced one at a time along the EmrE sequence (residues 3-110). Electron paramagnetic resonance spectroscopy (EPR) was used to characterize spin label dynamics, accessibility to the lipid and water phases as well as pairwise short-range proximity across the dimer interface (22-25). Changes in the EPR constraints upon substrate binding reveal movements in TM1 and TM2 and ordering of TM3 and the adjacent loop linking it to TM4. The compatibility between the EPR constraints and existing structures and models is evaluated to assess whether an antiparallel dimer is populated in liposomes.

**Experimental Procedures**

**Cloning and Site-Directed Mutagenesis.** The EmrE gene is cloned between the restriction sites \textit{NdeI} and \textit{BamHI} of plasmid pET15b⁺. The construct has a His₆ tag linked to the EmrE N-terminus by a thrombin sequence. The three native cysteines were mutated to alanines (C39A, C41A, and C95A) to yield a cysteine less EmrE hereafter referred to as WT*. Single cysteine mutants were generated for all amino acids spanning the first three transmembrane segments on pET15b⁺ WT⁺ background using the QuikChange method (Stratagene). Residues in the fourth transmembrane segment were cloned in pET21a⁺ WT⁺ between NdeI and HindIII. This construct has a spacer and a His₆ tag added at the C-terminus as previously described (26). All 110 cysteine mutants were also generated in pET20b⁺...
**WT* as described previously (27) for use in resistance phenotype activity assays. All the mutants were confirmed by sequencing.

**EmrE Expression, Purification, and Reconstitution.** The plasmids containing the EmrE single cysteine mutation were used to transform into BL21 (DE3) *E. coli* competent cells. The EmrE mutants were over-expressed in one liter minimal medium A (MMA) as described previously (27). Cells were grown at 37 °C until an OD of 1.4 was reached and protein expression was induced at 28 °C for most mutants by the addition of 1 mM IPTG. For mutants at positions 66 and 77, induction was carried out at 20 °C overnight. After 5 hours of induction, the cells were harvested by centrifugation, and resuspended in lysis buffer (27). The cells were then sonicated and homogenized using an EmulsiFlex-C5 (Avestin). The membrane pellets were solubilized with 1.5 % n-dodecyl-β-D-maltoside (β-DDM) in resuspension buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride, and 10 mM imidazole, pH 8). The solubilized membranes were ultracentrifuged at 388,000 g for 1 hour at 4 °C.

Solubilized EmrE was purified by Nickel affinity chromatography (27). The eluted EmrE mutants exhibiting dipolar broadening were underlabeled as follows. Mutants 14, 40, 44, 60 and 64 were spin labeled with 0.5-fold molar excess of MTSSL and incubated at room temperature for 1.5 hours after which a 20-fold molar excess spin label, every two hours (29). After 6 hours at room temperature, the samples were stored overnight at 4 °C.

Preparation of unilamellar liposomes. Asolectin (Avanti Polar Lipids, USA) dried from chloroform solution was resuspended in reconstitution buffer (50mM HEPES, 50mM sodium chloride, 2mM magnesium chloride, pH 7.5) and unilamellar vesicles were prepared by extrusion through 50nm Nuclepore Track-Etch Membrane filters (Schleicher and Schuell, USA). The protein sample was mixed at 1:500 to 1:1000 molar excess of preformed unilamellar liposomes. The mixture was incubated with gentle agitation at 4 °C for 2 hours, and then was diluted to a total volume of 10 ml with reconstitution buffer containing 50 mM HEPES, 50 mM sodium chloride, 2 mM magnesium chloride, pH 7.5. Bio-Beads SM2 (Bio-Rad) were added to the sample at the quantity of 80 mg per milliliter initial mixture and incubated for two hours at 4 °C, addition of fresh Bio-Beads was repeated twice at a two hour interval followed by overnight incubation at 4 °C. The reconstituted proteoliposomes were collected by ultracentrifugation at 388,000 g for 45 minutes at 4 °C. The pellets were then resuspended in reconstitution buffer.

**EPR Spectroscopy.** The EPR spectra of all reconstituted spin labeled EmrE mutants were collected on a Varian EPR spectrometer equipped with a loop-gap resonator at room temperature. Substrate binding to reconstituted EmrE was initiated by the addition of 16 mM of TPP + to ~100 μM of EmrE sample. The mixture was incubated at room temperature for 20 minutes. The samples were then drawn into round capillaries (VitroCom). The microwave power used was 2 mW incident, and the modulation amplitude was 1.6 G. Power saturation experiments were carried out on a Bruker ELEXSYS spectrometer equipped with a dielectric resonator (BrukerBiospin). Samples were loaded in gas-permeable TPX capillaries, and the measurements were carried out under nitrogen and in the presence of 20 % oxygen or 50 mM Nickel diamine diacetic acid (NiEDDA). The data were analyzed to obtain the parameter P1/2. The EPR accessibility parameter Π was calculated as previously described.

**Ethidium Resistance Assay.** The ability of mutants to confer resistance to ethidium bromide was tested as previously described (26). Overnight cultures of *E. coli* expressing mutant EmrE were
grown to saturation. The cultures were diluted 10-, 10³, and 10⁶-fold in fresh media, and 5 µl of each dilution was plated on LB-amp plates containing 200 µg/ml ethidium bromide. Growth was examined after incubation at 37 °C for 24 hours. The empty vector showed no growth at any dilutions and was used as negative control. EmrE WT* exhibited growth at all dilutions and was used as positive control. For all mutants, growth at 10³-fold dilution and above was considered active. Some mutants with growth at 0- and 10-fold dilutions were considered to have compromised activity. Finally, mutants with no growth at any dilution were considered inactive.

**TPP⁺ Binding Assay.** TPP⁺ binding experiments were carried out on an L-format Photon Technology International Fluorometer. The extent of binding of TPP⁺ was estimated by quenching of native intrinsic tryptophan (Trp) fluorescence emission (10). Samples containing 3 µM of spin-labeled EmrE mutants were mixed with 0, 6, and 12 µM TPP⁺ and incubated 20 minutes at room temperature. The samples were loaded in a 1 cm path-length quartz cuvette. Tryptophan emission spectra of the samples were recorded in the 310-350 nm range after excitation at 295 nm. For the high concentration experiments, the spin labeled EmrE samples were diluted to a final concentration of 20 µM and TPP⁺ was added to a final concentration of 80 µM and 120 µM.

**Results**

**Biochemical analysis of EmrE mutants.** To assess the transport activity of the EmrE cysteine mutants, we determined whether they conferred drug resistance on transformed *E. coli* cells. For this purpose, overnight cultures were diluted, spotted on agar plates containing 200 µg/ml Ethidium Bromide (EtBr), and allowed to grow for 24 hours at 37 °C (26). Bacterial growth was qualitatively characterized as described in the methods section. Figure 1 shows that most of the mutants are active at the highest dilution tested reflecting efficient EtBr clearance out of the cytoplasm. In contrast, a number of substitutions did not confer resistance at any dilution including at residues in TM1 (e.g. 10, 14, 17, 18) and TM3 (e.g. 58, 60, 63, 67) previously identified as important for substrate binding (10,11,30-32). Similarly, substitutions of glycine residues in TM4 (90, 97) lead to inactive transporters. For few sites, growth is observed at only one of the three dilutions suggesting compromised EmrE transport activity. Resistance reflects the balance between multiple factors including levels of EmrE expression and the competition between passive inward leaks of EtBr and its extrusion by the transporter. Thus, lack of resistance in some mutants may reflect changes in binding affinity as well as the kinetics of the transport cycle.

Therefore to complement the coarse phenotypic screen, we analyzed the substrate affinity of the purified, spin labeled proteins in dodecyl maltoside (ddm) micelles. EmrE trp fluorescence, specifically of trp 63, is quenched by binding of TPP⁺ (10). For each spin labeled mutant, the change in trp fluorescence upon addition of TPP⁺ was compared to that of the WT* (Supplementary Figure 1). Lack of change in trp fluorescence implies a reduction in affinity due to the cysteine substitution and/or spin labeling. The binding assay identified multiple residues in TM1-3 but not in TM4 with reduced affinity indicating that the latter is not directly involved in substrate binding and coordination. Mutations that reduce TPP⁺ affinity tend to cluster on the same face of each helix spaced by 3-4 residues and facing away from the lipid phase (see below). For a subset of sites such as 40, 44 and 60, trp quenching was restored at higher concentrations of EmrE and TPP⁺ suggesting that these mutants are capable of binding TPP⁺ albeit with lower affinity (Figure 2). The lower affinity is presumably responsible for the loss of resistance in the cell assay of Figure 1.

Most spin labeled mutants assembled into dimers with similar profiles to the WT* on size exclusion chromatography (data not shown). Where detected, changes in retention times reflect formation of larger oligomers rather than dissociation into a monomer as illustrated in Figure 3A for sites 90 and 97. Remarkably, the structural destabilization due to the substitution of these residues does not lead to loss of substrate affinity (Supplementary Figure 1).

The choice of detergent has a profound effect on the integrity of the EmrE dimer. Substitution of ddm with nonyl glucoside (NG), the detergent used in the crystal structure.
determination, alters apo-EmrE SEC profile particularly in pH 8 buffered-solutions. Overall, there is a significant loss in protein yield accompanied by a shift of the peak to retention times indicative of dissociation to a monomer (dashed line in Figure 3B). These results are in agreement with cross linking analysis in this detergent suggesting destabilization of the EmrE dimer (21). They are also consistent with the conclusion of Chen et al. that the crystal structure of apo EmrE in this detergent is likely to be misfolded (17).

Accessibility profiles identify transmembrane segments and water-accessible regions. The orientation and boundaries of EmrE transmembrane helices were deduced from measurement of collision frequency of each spin labeled site with paramagnetic probes that are either lipid soluble (molecular oxygen) or water-soluble (NiEDDA) (24,33). For a TM helix, the O₂ concentration gradient in the bilayer leads to a signature accessibility pattern consisting of 3.6 periodicity with increasing values at maxima near the middle of the membrane. This pattern is observed along TM1 while its modifications at the N-termini of TM2 and TM3 suggest specific structural features (Figure 4). Large Π(O₂) in the 30-35 stretch of residues along with an offset in the values at the minimum at residue 32 indicate loose packing of the N-terminus of TM2 and/or a tilt relative to the membrane normal. In contrast, low O₂ accessibilities and non helical periodicity at the N-terminus of TM3 (residues 58-64) reflect a sterically packed environment. TM3 emerges from these contacts at residues 65 and 66 where exposure to O₂ continues through the loop connecting TMs 3 and 4 and coincides with increased NiEDDA accessibility following residue 75.

The O₂ accessibility profile along helix 4 deviates significantly from that of a TM helix parallel to the membrane normal. Although the values at the maxima indicate direct contacts with the lipid phase, the pattern is asymmetric becoming successively smaller towards the C-terminus. The changes coincide with attenuated contrast between the two faces of the helix and an increase in the accessibility to the water soluble NiEDDA.

As expected, large NiEDDA accessibilities segregate predominantly to loops connecting the various TM segments. Compared to absolute values obtained in other membrane proteins such as MsbA (34), the EmrE accessibilities localize the loops to the membrane/water interface except for the 52-53 region. Measurable collisions with NiEDDA are observed at the termini of TMs 1 and 2 at sites of minimal oxygen exposure. They are 180° out of phase with O₂ indicating water penetration to the transmembrane segment presumably near the substrate binding site.

Spin label mobility identifies sites of tertiary contacts and residues at dimer interfaces. EPR spectra for spin labeled EmrE reconstituted in liposomes are shown in Supplementary Figures 2-5. The shapes of these spectra report the dynamic of the spin label side chain, i.e. its mobility, relative to the protein (22). The determinants of the rate and/or amplitude of spin label dynamics are the local steric crowding in the immediate vicinity of the spin label and the flexibility of the backbone to which it is attached. The spectra can also have contributions from broadening by short-range dipolar coupling between symmetry-related spin labels in the dimer.

Lineshapes characteristic of motionally restricted spin labels recur at sites within the TM helices with a characteristic 3.6 periodicity (Supplementary Figures 2-5). Together they define the surface of each TM that packs against other TM helices. On the opposite surface, the lineshapes reflect highly mobile spin labels as expected at lipid-exposed sites. This motif is observed along the entire lengths of TM1 and TM4 while TM2 and TM3 show distinct evidence of asymmetric packing. The N-terminal two turns of TM2 (residues 30-36) have uniformly mobile EPR spectra suggesting that this segment is lipid exposed with little tertiary contacts. In contrast, spin labels introduced at residues 56-60 of TM3 have restricted motion consistent with a sterically packed environment.

To quantitatively compare the mobility of spin labels, the inverse of the central linewidth ($\Delta H_0^{-1}$) was calculated from the EPR spectrum (Figure 5A). ($\Delta H_0^{-1}$) is a phenomenological
descriptor of mobility whose value was shown to correlate with the structural environment of the label (22). The 3.6 periodic variation in $(\Delta H_0)^{-1}$ (Figure 5A) reflecting alternation between buried, exposed and tertiary contact environments is consistent with four α-helical segments. The overall mobility pattern reflects the packing characteristic of each helix discussed above.

The EPR lineshapes at sites 14, 18, 40, 44, 60 and 64 have extensive broadening characteristic of strong dipolar coupling between spin labels separated by less than 15 Å. As previously noted in detergent micelles, the spectra reveal the presence of two populations, one in close proximity and one separated by more than 20 Å (19). Remarkably, these sites are located on the buried surfaces of TM1 (14, 18), TM2 (40, 44) and TM3 (60, 64) but not TM4. In TM1 and TM2 they occur near the middle of the helix. Analysis of labeling efficiency using an experimentally determined extinction coefficient demonstrates that the second population does not arise from incomplete labeling (27).

**Effects of TPP⁺ binding.** TPP⁺ binding leads to conformational changes that alter the environments of spin labels in TM1-3 as shown in Figure 5 and Supplementary Figures 2-5. In TM1, lineshape changes at residues N-terminal to E14 (8, 10 and 11) reveal an increase in motional restriction and order while those C-terminal to E14 such as 19, 21 and 22 near the C-terminus of TM1 report a decrease in steric restriction of the spin labels (Figure 6A and Supplementary Figure 2). In parallel, TPP⁺ binding eliminates dipolar coupling at residue 18 arising from the packing of the two spin labels at the dimer interface. Finally, oxygen accessibilities at residues 8 and 11 are dramatically reduced indicating transition of these residues from lipid exposed to buried environments (Figure 5B).

To establish the specificity of TPP⁺ induced conformational change, we took advantage of the absolute requirement of a glutamate at position 14 for substrate binding. For this purpose, the spectral changes at residues 10 and 18 were examined in a background where E14 was substituted with an alanine. Supplementary Figure 6 shows that the mutation eliminates the spectral sensitivity to the addition of TPP⁺ demonstrating the lack of bulk effects on the EPR lineshapes. In addition, the E14A mutation alters the EPR spectral lineshape eliminating dipolar coupling at site 18. This may reflect the transition of the transporter to a different conformational state as a result of the E14 substitution.

TPP⁺ binding induces opposite changes in O₂ and NiEDDA accessibilities at the two N-terminal turns of TM2 (Figure 5A and B). Spin labels at residues 31, 34 and 38, already in a lipid-exposed environment, report an increase in O₂ and a decrease in NiEDDA accessibility. This movement also affects the dipolar coupling between symmetry-related spin labels at sites 40 and 44 although the magnitude of the change is rather small (Figure 6B). The width of the broad component at site 40 is reduced (arrow in Figure 6B) while the dipolar splittings at site 44 become more prominent.

Starting at residue 56 and continuing through the N-terminal part of TM3, TPP⁺ binding uniformly reduces the mobility of spin labels (Figure 6B and C). This is consistent with the observation of a rigid loop connecting TM2 and TM3 in the EM structure (16, 35). At site 64, the strength of the dipolar coupling increases resulting in a spectrum similar to that of residues 14 and 40 indicating spin labels separated by less than 10 Å. Changes in O₂ accessibility are observed at a number of sites in TM3 and in the loop connecting TM3 and TM4 (residues 75 and 76).

In contrast, we did not observe significant TPP⁺-induced changes in the EPR lineshapes in TM4. Given previous evidence this helix does not participate in substrate binding (30, 36, 37), lineshape changes were screened in detergent micelles where EmrE has been shown to be functional (Supplementary Figure 5). The lack of spectral changes upon TPP⁺ binding was also confirmed in liposomes for a number of residues (Data not shown).

**Discussion**

An alternating access model of vectorial transport by EmrE posits transitions between at least four intermediates. The cycle begins by binding of protons (substrate) to an outward (inward)-facing conformation and ends at either of
the two putative apo-intermediates. Although
direct information regarding the conformational
changes that mediate transition between these
intermediates is lacking, most prevalent models
invoke reorientation of the binding site to shuttle
the protons and substrates between the two sides
of the membrane (35,38).

**Structure of apo EmrE.** Given a pKa of around 8.0
for the two E14 residues (12), the substrate-free
(apo) intermediate investigated here corresponds
to the protonated state. The EPR parameters
provide constraints on the local environment, the
orientation and tilt of the TM segments, and the
packing of the two monomers in the dimer. TPP+-
induced changes in these parameters reflect
structural rearrangements induced by substrate
binding.

The accessibility data clearly delineates
four helices as predicted by sequence analysis and
verified by the EM and crystal structures
(15,17,39). Each of TMs 1-3 is directly packed
against its counterpart in the dimer as deduced
from dipolar coupling between spin labels in these
segments. A recurrent theme in the corresponding
EPR lineshapes is their two-component nature
reflecting at least two spin label populations only
one of which is in close proximity. Because all
these mutants have similar SEC profiles to the
WT* with no evidence of dissociation, the second
population is likely to reflect a different packing
arrangement of the EmrE dimer. Remarkably,
weak dipolar coupling observed in TM4 (residues
96 and 97) is not consistent with either its packing
in the structures or its role as the dimerization
helix of the antiparallel dimer. Although the EPR
lineshapes along TM4 reveal a peripheral location
in direct contact with the lipid phase, the
O₂ accessibility pattern suggests that TM4 is on
average tilted relative to the membrane normal at a
steeper angle compared to the TPP+-bound EmrE
crystal structure.

**The TPP+-bound EmrE intermediate.** The simplest
interpretation of the changes in EPR parameters
along TM1 would involve a kink in the helix near
residue 14 allowing tighter packing of the turns N-
terminal to the critical E14 but reducing steric
contacts C-terminal to this residue along the lines
of an alternating access mechanism. This
interpretation, however, does not exclude the
possibility of more complex movements. For
instance, the increased steric restrictions at
residues 8 and 10 and the reduced dipolar coupling
at residue 18 caused by TPP⁺ binding may reflect
limited local helix rotation. The EPR data also
suggests tilting of TM2 at its two highly dynamic
N-terminal turns (residues 30-40) which are
already in direct contact with the lipid bilayer.
TPP⁺ binding changes the backbone structure of
TM3 and the two adjacent loops. O₂ accessibility
changes along TM3 indicate ordering of the
helical backbone in the TPP⁺ bound state (Figure
5B). Finally, the loop linking TM3 and TM4
becomes more lipid exposed, a rearrangement
likely to require a dynamic and flexible TM4.

**Comparison of the EPR parameters with the
structure of the TPP⁺-bound EmrE.** As noted
above, there are multiple EmrE regions where the
EPR parameters of the apo intermediate deviate
from the expected environments in the crystal
structure (17) and a model based on the EM
structure (35). These deviations may reflect the
conformational changes upon TPP⁺ binding or
arise from differences between the structure in
liposomes and in the crystal.

Overall the spectral broadening at sites in
TMs 1 and 2 can be reconciled with the packing
geometry of these segments in the crystal
structure. Although the buried environments of
these sites hinder a quantitative comparison, we
evaluated whether the pattern of proximity agrees
with the pairwise packing of helices in the crystal
structure by modeling the spin label in cones that
project along the Cα–Cβ bond (40). At residue 14
in TM1, the spin labels are expected to directly
point towards each other thus rationalizing the
broadened population. The projection of the side
chains at residue 18 is in agreement with the
absence of dipolar coupling in the TPP⁺-bound
state (Figure 6A). Thus, the observed spectral
broadening at this residue in the apo intermediate
reveals structural changes in the C-terminal region
of TM1 reducing the separation between the spin
labels and increasing spin label mobility at
residues 19, 21, and 22.

Residues 41 and 44 in TM2 are within the
range of dipolar coupling while at residue 40 one
of the labels is predicted to project away from the interface (Figure 6B). Therefore, comparison of the EPR data in the apo state with the TPP⁺-bound structure suggests structural changes in this part of TM2. A tilting motion away from the interface following TPP⁺ binding, predicted by the accessibility data, would reduce dipolar coupling at 40 as is experimentally observed.

Mapping the accessibility parameters onto the crystal structure provides a local perspective on the correspondence between the crystal structure and the EPR data (Figure 7). In general, the O₂ accessibility profile along TMs 1 and 2 is in reasonable agreement with the disposition of the two helices in the structure. Although the comparison is complicated by the structural asymmetry between the two monomers (see Figure 7), residues with large exposure to O₂ map to the lipid facing surfaces of TM1 and TM2. Because the EPR parameters represent an average from the two spin labels, deviations from the crystal structures are not unexpected.

The most pronounced disagreements between the EPR constraints and the crystal structure are in TM3 and TM4. Previously, dipolar coupling along TM3 (residues 60 and 75) was interpreted as indicative of parallel packing of the two TM3s at the dimer interface (19). With the finding that spin labels at residue 64 have strong dipolar coupling, the data could be brought into closer agreement with an antiparallel model if the N-terminus of TM3 is located well below the membrane/water interface, placing residues 60 and 64 in close proximity. We note, however, that the strong dipolar coupling predicted by the crystal structure at residue 63 was not observed experimentally. The proximities at residues 60 and 64 are not consistent with the EM-based model (35) where the N-terminus of TM3 are located at opposite end of the bilayer.

Moreover, there are striking discrepancies in the orientation of the TM3 C-terminus relative to the lipid phase. Residues of maximum O₂ accessibility (e.g. 74) are buried in the crystal structure while the minimas are lipid-facing in the crystal structure (Figures 4 and 7). Although NiEDDA accessibility is observed in the 75-80 loop (Figure 7B), relatively high O₂ accessibility in this region also suggests exposure to the hydrocarbon phase of the bilayer. Concurrent accessibility to O₂ and NiEDDA can only be rationalized by a highly dynamic backbone allowing large amplitude excursion of the loop between the aqueous and lipid phases.

As noted by Fleishman et al. (35), TM3 contains sequences, including a GXG motif (residues 65-67), that are likely to impart a degree of flexibility and account for a kink in the EM-based model. The lack of continuous periodicity in the O₂ accessibility may reflect a break in the helix which allows a dynamic C-terminal region to bend back towards the bilayer without perturbation of the tight packing at the N-terminus. The EPR data along the loop connecting TM3 and TM4 hints at large amplitude motion resulting in simultaneous accessibility to the bilayer and aqueous phases (Figure 8). An ensemble of conformations in this region may have an average EPR accessibility that deviates from the unique conformation trapped in the crystal.

While confirming that TM4 is in direct contact with the lipid bilayers, the EPR data implies a substantial tilt of its axis relative to the bilayer normal. The decrease in the values at successive maxima and the loss of helical periodicity indicate that after residue 103 the helix does not undergo stable tertiary contacts. This latter result is not inconsistent with the TPP⁺-bound crystal structure where the segments following residue 99 and 104 in each monomer are not resolved. The concomitant increase in NiEDDA accessibility at this turn suggests that this part of the helix is emerging out of the membrane (Figure 7B).

Concluding remarks. How does the EPR data weigh in on the dual topology controversy? As noted above, we find substantial deviations between the average liposome conformation and the x-ray and EM structures. Furthermore, the persistence of second populations in the dipolar-coupled spectra reveals conformations of EmrE with different packing interfaces. Nevertheless, the pattern of proximity along TM1 and TM2 suggests that one EmrE conformation is similar to the TPP⁺-bound crystal structure. Flexibility and dynamics are invoked to rationalize the
discrepancy between the EPR data along TM3 and its crystallographic conformation. The comparison is complicated by the low resolution of the latter which at 4 Å implies that residue assignment can be off by as much as a full helical turn. While noting this agreement, the definite test of the veracity of the crystal structure as a model of the antiparallel dimer requires verification of the helical packing in the monomer as well as long range distance measurements in the dimer (41). These experiments are currently in progress.

Vectorial transport by EmrE requires a re-orientation of the binding site from inward to outward-facing. The structural changes that underly the switch in accessibility have not been described experimentally but a model has been proposed on the basis of the available structures of TPP+-bound EmrE (35). Starting from the asymmetric dimer, changes in substrate binding site exposure require a simple interchange of the conformation of the two monomers. Consequently, the inward- and outward-facing conformations are linked by 180 rotation around the in-plane axis of symmetry. This conceptualization of alternating access implies no net change in residue environment, accessibility, or proximity across the dimer interface during the transition. Consequently, it does not predict changes in the EPR parameters as these are not affected by the symmetric exchange of spin label environments.

The results presented in this paper demonstrate that transition from the apo to the TPP+-bound intermediate does not follow the putative symmetric interchange described above. When mapped onto the crystal structure, the segments reporting changes in the EPR parameters appear to be distributed on both sides of the asymmetric dimer tracing a pathway through which the substrate may permeate. Specifically, the tilt at the N-terminus of TM2 provides direct access to the bilayer from which hydrophobic substrates may partition. A detailed global model of this conformational transition will also benefit from long range distance measurements (42).

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References
Figure Legends

Figure 1. Growth phenotypes of cells expressing EmrE single cysteine mutants. Saturated cultures of each mutant were diluted by a factor of $10^6$, $10^3$ and 10 and spotted on agar plates containing ethidium bromide as described in the methods section. The height of the bar indicates the maximal dilution at which cell growth was observed.

Figure 2. Changes in TPP$^+$ binding affinity of selected spin labeled EmrE detected by the level of trp quenching. For a number of residues (e.g. 44), binding required high concentration of EmrE and TPP$^+$ indicating reduction in affinity due to the cysteine substitution and/or attachment of the spin label.

Figure 3. Analysis of EmrE dimeric assembly by size exclusion chromatography. A) Spin labeling of selected residues in TM4 leads to changes in the retention time indicative of aggregation. B) The detergent nonyl glucoside (NG) destabilizes the apo EmrE resulting in dissociation to a monomer. The inset is a cut out from an SDS-PAGE confirming the protein identity in each SEC peak.

Figure 4. Accessibility profiles of apo EmrE. Red $\Pi(O_2)$, blue $\Pi(NiEDDA)$. Both parameters show periodic variation as a function of residue number in the regions of the TM helices. The dashed lines indicate helix and loop boundaries on the basis of the crystal structure assignment.

Figure 5. Sequence-specific environmental parameters in the apo (color) and TPP$^+$ bound intermediates (gray). A) Mobility parameter ($\Delta H_0)^{-1}$, B) Accessibility to molecular O$_2$, and C) Accessibility to NiEDDA. The regions of accessibility changes are highlighted in yellow.

Figure 6. EPR lineshape changes upon TPP$^+$ binding reveal changes in packing and proximity along the interfaces of TMs1-3. For each panel, a close up view of the structure highlighting the spin labeled residues is shown. In panels A and B, the bound TPP molecule is also visible. All spectra were normalized to the same number of spins and then scaled to reveal the details of the lineshapes.

Figure 7. A) $\Pi(O_2)$ and B) $\Pi(NiEDDA)$ mapped onto a ribbon representation of the two EmrE monomers. The second monomer is shown in a surface rendering. Selected residues from each TM are shown to provide markers and highlight the structural asymmetry between the two monomers.

Figure 8. Model of TM3 based on the EPR data (yellow) superimposed on a ribbon representation of the helix in the crystal structure. It assumes a two-fold symmetry axis near the middle of the bilayer. This configuration satisfies the constraints of proximity between residues 60 and 64. The arrows indicate
movement of the C-terminal tail that allows residues at the C-terminus and in the loop linking TM3 to TM4 to sample the membrane and aqueous environments.
Figure 1
Figure 2
Figure 3
Figure 7

(A) 

(B)
Structure, dynamics and substrate-induced conformational changes of the multidrug transporter emre in liposomes

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