New free-exchange model of EmrE transport

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EmrE is a small multidrug resistance transporter found in *Escherichia coli* that confers resistance to toxic polyaromatic cations due to its proton-coupled antiport of these substrates. Here we show that EmrE breaks the rules generally deemed essential for coupled antiport. NMR spectra reveal that EmrE can simultaneously bind and cotransport proton and drug. The functional consequence of this finding is an exceptionally promiscuous transporter: not only can EmrE export diverse drug substrates, it can couple antiport of a drug to either one or two protons, performing both electrogenic and electroneutral transport of a single substrate. We present a free-exchange model for EmrE antiport that is consistent with these results and recapitulates Δ pH-driven concentrative drug uptake. Kinetic modeling suggests that free exchange by EmrE sacrifices coupling efficiency but boosts initial transport speed and drug release rate, which may facilitate efficient multidrug efflux.

NMR | membrane protein | multidrug transport | coupled transport | protein dynamics

Secondary active transport moves one substrate across a membrane against its concentration gradient by coupling it to downhill transport of a second substrate, often a proton. This coupled transport process may move both substrates in the same direction (symport) or in opposite directions (antiport). To move molecules across the membrane, the substrate binding site must be alternately accessible to either side of the membrane. Symport or antiport of two substrates is generally explained using models that restrict this alternating access of the transporter to specific states (Fig. 1). These models are appealing because they provide a simple mechanism that efficiently couples transport of the two substrates.

Here we investigate the mechanism of proton/drug antiport by the small multidrug resistance (SMR) transporter, EmrE. EmrE uses the proton motive force (PMF) across the inner membrane of *Escherichia coli* to drive efflux of toxic polyaromatic cations, conferring resistance to these compounds. The single binding pocket of EmrE is defined by two glutamate 14 residues (1–3), one on each of the two monomers in the asymmetric homodimer (4–7). This binding site can accommodate one drug substrate or up to two protons. Alternating access of the asymmetric homodimer is achieved by a conformation swap between the two monomers (5) and is necessary for transport activity (8). Traditionally, EmrE antiport has been explained by the pure-exchange model (9) in Fig. 1A. Such “pure exchange” of one drug for two protons with no slippage results in tightly coupled stoichiometric antiport. This is achieved by: (i) limiting substrate binding such that both substrates never bind simultaneously, and (ii) limiting in/out exchange (alternating access) to substrate-bound states (fully protonated or drug-bound). Several lines of evidence support this model. Competition between drug and proton binding is demonstrated by substrate-induced proton release (10, 11), a decrease in substrate-binding affinity at low pH (1), and a bell-shaped pH-dependence of transport activity (3). In addition, observation of electrogenic transport of monovalent, but not divalent, substrates is consistent with a 2:1 H^+ /drug⁺ transport stoichiometry (12). Other transport mechanisms have been considered previously (11), but the traditional pure-exchange model has been favored in

the absence of compelling data to justify selection of a more complex scheme.

However, the highly dynamic nature of EmrE (13–15), critical for its ability to transport diverse substrates, is hard to reconcile with the strict limitations on alternating access in the pure-exchange model. Recent NMR data have provided evidence that EmrE violates at least the second stipulation of the traditional model: protonation of drug-free EmrE is asymmetric (16), such that a singly protonated state exists near neutral pH, and all of the protonation states (2 H^+ -bound, 1 H^+ -bound, empty) engage in alternating access (16, 17). These findings suggest the need to develop a new model for EmrE transport activity. In this study, we use NMR spectroscopy and liposomal flux assays to test the pure-exchange model of EmrE antiport. We show that EmrE violates both requirements of pure-exchange antiport and utilizes multiple proton/drug transport stoichiometries. We developed a “free-exchange” EmrE antiport model which reconciles the novel states and unrestricted alternating access behavior of EmrE with its well-established proton-driven drug efflux activity. Our model accounts for previously inexplicable behaviors of EmrE, such as the ease of converting the protein to a symporter. Furthermore, our model suggests that a reduced coupling efficiency may be a necessary price for the structural flexibility required to bind and efficiently efflux diverse substrates.

Significance

EmrE facilitates *Escherichia coli* multidrug resistance by coupling drug efflux to proton import. This antiport mechanism has been thought to occur via a pure-exchange model, which achieves coupled antiport by restricting when the single binding pocket can alternate access between opposite sides of the membrane. We test this model using NMR titrations and transport assays and find it cannot account for EmrE antiport activity. We propose a new free-exchange model of antiport with fewer restrictions that better accounts for the highly promiscuous nature of EmrE drug efflux. This model expands our understanding of proton-coupled transport and has implications for both transporter design and drug development.

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Fig. 1. Models of tightly coupled secondary transport. (A) The pure-exchange model of coupled antiport applied to EmrE (red hexagon, TPP⁺). The asymmetric homodimer of EmrE is represented by the distinct shapes of the two conformations, and the two monomers are colored blue and magenta. (B) General model for proton-coupled symport illustrating the differences in which states participate in alternating access. Gray structures represent intermediates which are restricted from alternating access in these models.

Results

Substrate Binding Is Not Exclusive. Competition between drug and proton binding to EmrE is well-established, but the data do not prove mutually exclusive binding, a stipulation of the pure-exchange model (1, 10, 11). The recent demonstration of asymmetry in proton binding (16) led us to reconsider whether EmrE can bind a drug and proton simultaneously. To test this, we performed NMR pH titrations of EmrE saturated with the drug substrate tetraphenylphosphonium (TPP⁺) and solubilized in isotropic bicelles. We have previously shown that E14 and H110 are the only residues in EmrE that titrate near neutral pH. Furthermore, titration of E14, but not H110, leads to a global conformational change in the protein that is evident in an NMR pH titration (16). If proton and drug binding are exclusive, as predicted by the traditional model of EmrE antiport, then TPP⁺-saturated EmrE should not bind any protons and thus will not titrate globally with pH. However, many peaks in the NMR spectrum do titrate with pH (Fig. 2A and B and *SI Appendix*, Fig. S1), demonstrating that protonation does occur when TPP⁺ is bound. In contrast to the two protonation events observed for drug-free EmrE (pK_a values 7.0 ± 0.1 and 8.2 ± 0.3) (16), the linear movement of the peaks in the TPP⁺-bound NMR pH titration is consistent with a single protonation event with a pK_a of 6.8 ± 0.1 (Fig. 2C and *SI Appendix*, Fig. S2). To test whether this protonation occurs on the critical E14 residue, we repeated the pH titration with TPP⁺-saturated E14D-EmrE, which has a lower pK_a but still binds TPP⁺ (1–3, 16). As expected, we observed a shift in the titration midpoint to lower pH, reflecting the lower pK_a of E14D-EmrE (Fig. 2A and B and *SI Appendix*, Fig. S3) and confirming we are monitoring protonation of E14 in drug-bound EmrE.

It is well known that TPP⁺ and H⁺ binding is competitive such that the TPP⁺ binding affinity of EmrE is weaker at low pH (1). Thus, it is important to ensure that the pH-dependent chemical-shift changes are not due to pH-dependent loss of substrate binding. We used isothermal titration calorimetry (ITC) to measure TPP⁺ binding at low pH. In isotropic bicelles at 45 °C (matching the NMR sample conditions), the K_d^{apparent} for TPP⁺ is $70 \pm 9 \mu\text{M}$ at pH 5.5 (*SI Appendix*, Table S1). With this affinity, >95% of EmrE will remain TPP⁺-bound in the NMR sample at pH 5.5. As a second more direct test, we performed an NMR-monitored TPP⁺ titration at pH 5.2. As expected, there were no changes in the peak positions with increasing TPP⁺ concentrations (*SI Appendix*, Fig. S4), only changes in peak intensity. Therefore, the pH-induced chemical-shift changes in the NMR spectra are not due to loss of TPP⁺ binding. Loss of peak

Fig. 2. EmrE can bind drug and proton simultaneously. (A) NMR pH titrations of TPP⁺-bound ²H/¹⁵N- WT (45 °C and 35 °C) and E14D (35 °C) EmrE shown for residue Ala10, a well-resolved residue near E14 (full spectra in *SI Appendix*, Figs. S1 and S3). pH values range from 5.2 (pink) to 8.3 (purple) for WT and 4.0 (yellow) to 8.0 (navy) for E14D-EmrE. (B) Chemical shifts of Ala10 plotted versus pH highlight the difference between WT and E14D-EmrE and the asymmetric response of monomer A and B. Lines represent global pK_a fit. (C) Global fitting of the pH-dependent chemical shifts from monomer B residues near E14 (solid symbols) yields a single pK_a of 6.8 ± 0.1 at 45 °C. Monomer A residues (open symbols) have relatively pH-independent chemical shifts. Error bars are smaller than the symbols. (D) Plotting the chemical shift changes ($\Delta\omega$) between pH 5.6 and 7.6 onto the structure of TPP⁺-bound EmrE (PDB 3B5D) highlights the localization of pH-dependent effects in monomer B (gray, residues not resolved at both pH values). E14 is shown as red sticks.

intensity with no change in peak position at lower TPP⁺ concentrations reflects “slow exchange” on the NMR timescale. In contrast, the change in peak position observed as a function of pH reflects “fast exchange.” This rate difference is not surprising and results in distinct spectral changes in the NMR spectra that allow these two events to be readily distinguished. This NMR data shows that EmrE can simultaneously bind a drug and a proton at physiological pH.

Substrate Binding Is Asymmetric. Mapping the NMR data onto the structure reveals a broad distribution of residues that sense E14 protonation in TPP⁺-bound EmrE (Fig. 2D), consistent with protonation-dependent conformational changes in drug-bound EmrE. This is not surprising given the protonation-dependent transport activity of EmrE (3) and the coupled structural and dynamic changes that occur upon protonation in the absence of substrate (16–18). Interestingly, residues corresponding to monomer B in the asymmetric homodimer have much larger chemical-shift changes upon pH titration of TPP⁺-bound EmrE (Fig. 2D). This implies that TPP⁺ interacts asymmetrically with EmrE, associating with the E14 on monomer A, such that protonation of this residue is prevented, while the E14 on monomer B remains accessible for protonation. This is further supported by the TPP⁺ titration at low pH, which shows that residues in monomer A are more sensitive to TPP⁺ binding (*SI Appendix, Fig. S4*). Such asymmetric interaction of the substrate with the two monomers was suggested by the cryoEM structure of TPP⁺-bound EmrE (6). It is also consistent with the asymmetric structure of the EmrE homodimer, the unique chemical shifts of the two E14 residues (19), and the asymmetric protonation of the two E14 residues in drug-free EmrE (16).

Drug Binding only Releases One Proton at Low pH. To validate this extraordinary finding that an antiporter can simultaneously bind both substrates, we measured TPP⁺-induced proton release from EmrE at low pH. According to the pure-exchange model, substrate binding is mutually exclusive such that both protons should be released from the EmrE homodimer upon TPP⁺ binding at low pH. In contrast, if EmrE is able to bind TPP⁺ and a proton simultaneously, TPP⁺ binding will only trigger release of a single proton per dimer at low pH. A previous measurement of TPP⁺-induced proton release was inconclusive because the amount of TPP⁺ added was insufficient to saturate EmrE at low pH (10). We repeated this measurement with EmrE solubilized in isotropic bicelles using a saturating TPP⁺ concentration and observed release of 1.2 ± 0.2 protons per EmrE dimer at pH 5.5 (Fig. 3A and *SI Appendix, Fig. S5*). Because the weak buffering required for direct proton detection in this assay results in relatively large errors, we used a second experimental approach to verify the results. By measuring TPP⁺ binding with ITC in multiple buffers with different heats of ionization (20), we could determine the number of protons released per TPP⁺ binding event and confirm binding saturation (Fig. 3B and *SI Appendix, Table S1*). We again detected 1.2 ± 0.1 protons released per dimer. The ITC data also confirmed the known 1:1 TPP⁺/dimer binding stoichiometry remains at low pH. These proton release values are much closer to 1 than 2, consistent with the NMR data demonstrating simultaneous binding of 1 TPP⁺ and 1 H⁺ to the EmrE dimer at low pH.

EmrE Alternates Access When Bound to both Substrates. The ability of EmrE to bind a drug and proton simultaneously will only affect net transport if this state engages in alternating access. For EmrE, the two monomers within the asymmetric homodimer swap conformations to switch between open-in and open-out (alternate access), and this rate can be quantitatively measured using TROSY-selected ZZ exchange NMR experiments (5, 21). We compared the rate of alternating access for TPP⁺-saturated

Fig. 3. TPP⁺-induced proton release at low pH. (A) Direct pH measurement of the nanomoles H⁺ released upon addition of saturating TPP⁺ as a function of EmrE concentration yields a slope of 1.2 H⁺ released per EmrE dimer. Dotted and dashed lines show expected behavior if one or two protons are released per dimer, respectively. The estimated error in the protein concentration was 10%. Vertical error bars represent SE determined from replicates. (B) ITC was used to characterize TPP⁺ binding to EmrE in buffers with different ionization enthalpies (ΔH_{ion}). The relationship between ΔH_{obs} (per EmrE monomer) from each ITC experiment and ΔH_{ion} of each buffer is linear with a slope of 1.2 H⁺ released per dimer.

EmrE in isotropic bicelles at high pH (only drug-bound) and low pH (both drug- and proton-bound) and found they were nearly identical. (Fig. 4 and *SI Appendix, Fig. S6*). Thus, EmrE can move both substrates across the membrane at the same time, violating the expected behavior of an antiporter, and requiring the development of a new transport model.

Importantly, chemical shift reflects the unique environment of each nucleus within the 3D structure of the protein. Thus, the peaks in the NMR spectra correspond to the two distinct monomer conformations (shapes in Fig. 5A), not monomer identities (colors in Fig. 5A). The asymmetric response of the residues in each monomer to TPP⁺ and pH in the NMR experiments show that TPP⁺ interacts more closely with the monomer in conformation A, while protonation of E14 can still occur on the monomer in conformation B. Since the two monomers swap conformations during alternating access, this means that TPP⁺ (and H⁺) is (are) swapped back and forth between the two monomers as part of the alternating access process, as illustrated in Fig. 5A. Therefore, although only one E14 is necessary for TPP⁺ binding, the swapping of substrate between monomers during alternating access will require both E14 residues for transport. This is consistent with the “functional symmetry” of E14 (22), the observation of TPP⁺ binding to mixed WT-E14C EmrE heterodimers (7), and the dominant-negative phenotype of E14 mutants *in vivo* and *in vitro* (23).

A Free-Exchange Model for EmrE Transport. To accommodate the new states and transitions of EmrE, we expanded the transport scheme (Fig. 5A), removing the restrictions on simultaneous substrate binding and alternating access that are imposed by the pure-exchange model. The model predicts that EmrE should have multiple transport pathways, including the well-established 2:1 H⁺/drug⁺ antiport (12) (Fig. 5B) as well as 1:1 antiport, proton leak, uncoupled uniport of drug, and even symport (Fig. 5C). The rates of individual steps in the model are similar, so flux should occur through multiple pathways. Partitioning between the transport pathways will be determined by the relative rates of individual steps, making kinetics an important factor in determining the net stoichiometry of the transport process. Furthermore, because the multiple transport pathways move different net charges across the membrane, they will be driven by distinct thermodynamic driving forces. Kinetics is therefore an important factor controlling net flux, which will vary depending on the precise conditions.

To understand if it is possible to produce the well-established proton/drug antiport activity of EmrE with this scheme, we performed mathematical simulations. We can estimate all of the rate

Fig. 4. All EmrE substrate-bound states engage in alternating access. The composite peak ratio from ^1H - ^{15}N TROSY-selected ZZ-exchange NMR experiments is shown for multiple residues at pH 5.2 (pink circles), pH 7.0 (green diamonds), and pH 8.0 (blue squares). Global fits yield alternating access rates of $8.9 \pm 1.2 \text{ s}^{-1}$ at pH 5.2 (TPP $^+$ /H $^+$ both bound) and $7.3 \pm 0.7 \text{ s}^{-1}$ at pH 8.0 (only TPP $^+$ -bound). Error was estimated with jackknife analysis of individual residue fits (dotted lines).

constants from our own and others' experimental data (*SI Appendix, Fig. S7 and Table S2*). Rates of alternating access are based on NMR dynamics measurements (5, 16, 17). TPP $^+$ on- and off-rate estimates were determined previously for detergent-solubilized EmrE (11). We assumed fast proton on-rates ($\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) and used the pK_a values determined by NMR (this work and ref. 16) to estimate off-rates. These are reasonable assumptions given the small size of H $^+$, the relatively fast on-rates for TPP $^+$ binding, and the fact that binding affinities of drug-substrates are primarily determined by the off-rate (11).

Numerical simulation of ΔpH -driven transport (*SI Appendix, Appendix 1*) results in rapid concentrative uptake of TPP $^+$ into liposomes (Fig. 6*A* and *B*). A 2-fold pH gradient drives 80-fold concentration of TPP $^+$, a reduction in coupling efficiency compared with the pure-exchange model with a strict 2H $^+$:1TPP $^+$ transport stoichiometry. These results clearly demonstrate that ΔpH -driven coupled antiport of TPP $^+$ can be achieved in our free-exchange model without restricting alternating access or substrate binding.

To better understand how coupled antiport occurs in our model, we developed a single-molecule Gillespie simulation (24) (*SI Appendix, Appendix 2*). Unlike the previous deterministic simulation, this stochastic simulation ignores the finite liposomal volume, but informs on the relative frequency of each transition, which is indicated by arrow thickness in Fig. 6*C*. Interestingly, TPP $^+$ is most likely to bind proton-free EmrE but be released subsequent to protonation. Simultaneous proton and TPP $^+$ binding enhance the release of TPP $^+$ (*SI Appendix, Fig. S7*), resulting in faster turnover. This mechanism may be advantageous for a multidrug transporter because it allows efficient release of substrates with a wide range of affinities.

While our simulations demonstrate coupled antiport, the model will still need to be tested and revised. Several rate constants were estimated using simplifying assumptions, and all were measured using solubilized EmrE. Importantly, solubilization results in a symmetric environment rather than the asymmetric conditions experienced in a membrane under a proton motive force. Furthermore, our model does not yet account for a membrane potential, as it remains unclear precisely how a membrane potential

affects discrete steps of transport pathways (25). Because the multiple transport pathways in our model move different net charges across the membrane, the membrane potential will affect the partitioning between pathways in a manner that is currently unaccounted for by our model. Nevertheless, the ability of our simulation to qualitatively recapitulate EmrE antiport is noteworthy because it breaks long-standing assumptions about the mechanism of coupled antiport.

Unlike the pure-exchange model, our model allows free exchange of the transporter, with alternating access permitted in all states. This has significant functional implications because it allows for free exchange of substrates with multiple transport stoichiometries. This differs dramatically from the single antiport stoichiometry achieved with pure exchange of substrates across the membrane. To test our free-exchange model, we experimentally investigated two of its key predictions: (i) Does EmrE leak protons? (ii) Is EmrE a strictly-coupled 2:1 H $^+$ /drug $^+$ antiporter?

EmrE Does Not Rapidly Leak Protons. To test whether EmrE leaks protons, we created EmrE proteoliposomes with a negative inside-membrane potential. Protons should rapidly enter the liposome under these conditions if EmrE leaks protons, increasing the weakly buffered external pH. However, no significant proton movement was detected (Fig. 7*A* and *B*), demonstrating that drug-free EmrE does not rapidly leak protons across the membrane. Addition of a protonophore did result in rapid pH change, confirming the integrity of the assay. These results contrast with the prediction of a rapid proton leak from the NMR exchange rates. However, all of the rates in the free-exchange model were measured with symmetric conditions ($\Delta\text{pH} = 0$, $\Delta\psi = 0$). In contrast, this assay is performed in the presence of a membrane potential. Perhaps the asymmetry introduced by the membrane potential alters the structure or dynamics of EmrE in a way that prevents proton leakage.

EmrE Does Not Have a Strict 2:1 H $^+$ /Drug $^+$ Antiport Stoichiometry. To determine whether EmrE can transport drug substrates with different proton-coupling stoichiometries, as predicted by the

Fig. 5. Free-exchange model for EmrE transport. (A) Three-dimensional transport scheme including all known states and transitions of EmrE based on NMR and biophysical assays. (B) Orange arrows highlight a 2:1 H $^+$ /Eth $^+$ antiport pathway in the direction observed during liposomal transport assays. (C) Green arrows highlight the possibility of 1:1 H $^+$ /Eth $^+$ symport under the liposomal assay conditions.

Fig. 6. Simulation of the kinetically driven free-exchange model. (A) Cartoon illustrates the ΔpH -driven liposomal loading that was modeled. (B) Kinetic simulation of TPP^+ uptake into liposomes using the rate constants in *SI Appendix, Fig. S7 and Table S2* results in proton-driven concentrative uptake of TPP^+ into EmrE proteoliposomes (pH 6 inside), when $\Delta\text{pH} = 1$ (pH 7 outside, dashed lines) or $\Delta\text{pH} = 2$ (pH 8 outside, solid lines). When $\Delta\text{pH} = 0$ (dotted line, expanded below), 50 nM TPP^+ rapidly equilibrates. (C) Arrow line weights on the transport scheme reflect the relative frequency of each transition during the Gillespie simulation (pH 8 out, pH 6 in, 50 nM TPP^+ outside); darker lines indicate higher frequency.

NMR data, we performed transport assays. We reconstituted EmrE into 3:1 POPC/POPG liposomes and observed transport by directly detecting proton flux in the weakly buffered solution outside of the liposome. We first performed transport assays using TPP^+ (*SI Appendix, Fig. S8 and Table S4*), but these assays are complicated by the high affinity of TPP^+ for EmrE as well as its hydrophobicity and membrane permeability. We therefore compared ΔpH - and $\Delta\psi$ -driven transport of ethidium (Eth^+), an EmrE substrate that is more well-suited to transport assays due to a weaker binding affinity [K_d at least 1,000-fold weaker (26)] and decreased hydrophobicity compared with TPP^+ .

We first performed ΔpH -driven transport assays. The proteoliposomes were reconstituted in 100 mM MES, 100 mM KCl, pH 6 to create a strongly buffered interior solution. They were then desalted into weak buffer for pH monitoring (0.5 mM MOPS, 100 mM KCl, pH 6) and the external pH was adjusted to pH 8 (Fig. 7 C and D). Adding drug to the external solution results in EmrE-mediated drug uptake into the liposomes coupled to proton efflux down the pH gradient (Fig. 7D and *SI Appendix, Table S3*). An electrogenic transport process, such as $2\text{H}^+/\text{Eth}^+$ antiport, will proceed under these conditions until the net charge movement creates an opposing membrane potential sufficient to stop further transport. However, due to the relatively large capacitance of the membrane, a significant amount of electrogenic transport can occur before an opposing membrane potential is achieved. Subsequent addition of the potassium ionophore valinomycin, which enables flux of K^+ to relieve any membrane potential, results in additional transport (Fig. 7 D and H and *SI Appendix, Table S3*). This demonstrates that a membrane potential had developed and supports the well-established $2\text{H}^+/\text{drug}^+$ antiport activity of EmrE (12). Two control experiments confirm that we are observing EmrE-mediated proton efflux and not simply proton release upon drug binding: (i) inactivating EmrE with DCCD (3) inhibits the proton release from the proteoliposomes and (ii) doubling the number of EmrE dimers per liposome (by doubling the EmrE:lipid ratio), while keeping the total number of liposomes constant results in identical proton release from the proteoliposomes (Fig. 7 D and H and *SI Appendix, Table S3*). Binding-related proton release depends on protein concentration and would double, while trans-

port depends only on substrate concentrations and liposome volume, which are the same. Besides establishing the functionality of EmrE, these ΔpH -driven assays demonstrate that Eth^+ -bound EmrE does not rapidly leak protons: when the protonophore CCCP is added at the end of the assay, a large pH change is recorded, demonstrating that the pH gradient was maintained throughout the assay. Together with the data in Fig. 7B, this shows that EmrE does not rapidly leak protons in either the drug-free or drug-bound states. Thus, 1:1 antiport combined with uncoupled proton flux cannot account for the observed increase in flux after addition of valinomycin.

Assuming a strict 2:1 antiport stoichiometry, the measured total proton release corresponds to transport of only 38 nmol of Eth^+ . Using simple estimates of the liposome internal volume, this corresponds to concentration of Eth^+ inside the liposome by ~60- to 70-fold, much less than expected for strict $2\text{H}^+/\text{Eth}^+$ antiport driven by $\Delta\text{pH} = 2$ (see *SI Appendix, Appendix 3* for detailed calculations). This contradiction could be explained if EmrE transport activity is a mixture of 2:1 and 1:1 antiport stoichiometries. However, because accurate quantitation is difficult (see *SI Appendix, Appendix 3* for more extended discussion), we turned to $\Delta\psi$ -driven transport assays, which more robustly distinguish different transport stoichiometries.

We compared EmrE transport activity under conditions of positive inside, neutral, and negative inside liposomes (Fig. 7 E–H and *SI Appendix, Table S5*). To generate the membrane potentials ($\Delta\psi$), we reconstituted EmrE into proteoliposomes with 100-fold K^+ gradients (100 mM KCl vs. 1 mM KCl + 99 mM NaCl). Upon addition of valinomycin, K^+ diffusion down the gradient creates either a positive inside (low K^+ inside) or a negative inside (high K^+ inside) membrane potential (Fig. 7 A and E). When the internal and external K^+ concentrations are matched, no membrane potential is generated. In all cases, pH ~ 7.5, $\Delta\text{pH} = 0$, and the interior was strongly buffered (50 mM MOPS), while the exterior was weakly buffered (0.05 mM MOPS) to allow direct monitoring of H^+ flux. These assays confirm the previous proton leak assay: addition of valinomycin to generate a membrane potential results in only a slight pH drift (Fig. 7 F and G). It does not result in significant pH change, as would occur if H^+ could leak through EmrE in response to the potential. When Eth^+ is added to the external solution of $\Delta\psi = 0$ proteoliposomes, the external pH decreases due to coupled influx of Eth^+ and efflux of protons by EmrE (Fig. 7F). This is driven entirely by the Eth^+ gradient since ΔpH and $\Delta\psi = 0$ (Fig. 7E). When the assay is repeated with a positive inside $\Delta\psi$, proton efflux is enhanced (Fig. 7F). Because a positive inside $\Delta\psi$ can only drive electrogenic transport, these data supports 2:1 H^+/drug^+ EmrE antiport activity (Fig. 7E). Such positive inside-driven EmrE antiport has been observed previously by monitoring drug loading directly (12). Our data confirm that the same result is obtained when monitoring the antiported protons. Control experiments (Fig. 7F) with an inactive mutant, E14Q-EmrE, show no proton flux, again confirming that the pH changes are due to transport through EmrE.

We then tested the effect of a negative inside membrane potential (Fig. 7 E and G) and obtained an entirely unexpected result. The Eth^+ concentration gradient will always favor Eth^+ influx, and thus H^+ efflux (external pH decrease) due to the coupled antiport activity of EmrE. The negative membrane potential will oppose 2:1 H^+/Eth^+ antiport, just as a positive membrane potential favors it (Fig. 7E). We therefore expected to observe a small decrease in external pH, somewhere between zero and the ΔEth^+ -driven transport observed for $\Delta\psi = 0$ liposomes. The exact value would depend on the balance between ΔEth^+ , $\Delta\psi$, and the net stoichiometry, since 1:1 H^+/Eth^+ antiport would not be inhibited by the membrane potential. Instead, upon addition of Eth^+ to negative inside proteoliposomes, the external pH increased, indicating proton movement into the liposomes (Fig. 7G). Because Eth^+ is only added externally, this

and then the membrane potential drives efflux with a 2:1 stoichiometry, twice as many protons would be imported into the liposome than released from the liposome and the external pH would rise beyond the initial value. However, this is not consistent with the results of the experiment where Eth^+ was added first followed by valinomycin (Fig. 7G). While the data from these transport assays cannot definitively differentiate between this third option or EmrE symport, symport is the most likely explanation for the data. In either case, it is clear that strict 2:1 antiport cannot account for the EmrE-dependent transport activity observed. In fact, the lag phase observed when Eth^+ is added to the negative inside liposomes is expected if there are multiple transport pathways, since the different pathways will have different driving forces and kinetics. Whatever the microscopic pathways, our results indicate net symport of drug and proton into liposomes by EmrE in response to a negative inside-membrane potential. Together with the data from our positive inside-liposome assay demonstrating 2:1 H^+/Eth^+ antiport activity, these results suggest that EmrE must be able to perform proton-coupled drug transport with multiple transport stoichiometries.

Discussion

Reassessing the Mechanism of Secondary Active Transport. Advances in *in vitro* studies of transporter function and structure have expanded our understanding of the range of conformational changes that can produce alternating access (27, 28), and the mechanisms controlling stoichiometry and transport (29). MdfA and PepT_{ST} have different proton/substrate transport stoichiometries when transporting substrates of different size or charge (30, 31) and the stoichiometry of sugar/proton symport by LacY is pH-dependent (32). The CLC-ec1 transporter performs 2:1 H^+ /substrate-coupled antiport of some anions and uncoupled transport of other anions (33). However, the CLC transport mechanism is not a standard alternating access model; it relies on relatively small movements of amino acid side chains that are easily altered by binding different ions (33, 34). This contrasts with EmrE, where the crystallographic (4), cryoEM (35), and NMR data (5, 8) all support a standard alternating access mechanism with large-scale changes in helix orientation. Furthermore, the CLC family evolved to include both channels and exchangers, and the observed uncoupling and leak behavior, appears to shift the protein function along this continuum between transporter and channel for different substrates. In contrast, EmrE never appears to have channel-like behavior: pH gradients and membrane potentials are never fully dissipated in our liposomal assays. Thus, we have described EmrE as having multiple transport stoichiometries due to the multiple transport pathways in the free-exchange model, rather than a single transport stoichiometry plus leak or uncoupled transport.

Here we show that EmrE can transport a single substrate with multiple proton/substrate transport stoichiometries. This flexibility of transport stoichiometry is achieved by a number of unexpected features. First, TPP^+ binds asymmetrically in the active site, making space for a proton to bind simultaneously. Second, all of the EmrE proton- and drug-bound states are capable of engaging in alternating access. These phenomena of mutual binding and unlimited alternating access at first seem to jeopardize EmrE's capacity for coupled antiport, but a closer look reveals the efficacy and benefits of such a mechanism. These features of EmrE are reminiscent of the *de novo* designed transporter, Rocker (36), although EmrE is more efficiently coupled. It suggests that functionally coupled transport can be achieved without the need to invoke significant constraints on the states and transitions of the transporter, perhaps providing new insights for the rational design of *de novo* transporters.

How is antiport driven with these unique parameters? In this free-exchange model, the relative rates of the individual steps will determine the efficiency of proton-coupled transport. Although H^+ and TPP^+ can bind simultaneously, their binding is

still negatively linked, resulting in differential TPP^+ affinity for the open-in and open-out states of EmrE in the presence of a transmembrane pH gradient. In fact, a pH gradient may even skew the equilibrium between the open-in and open-out states (17). Such differential substrate affinity is important for determining the relative kinetics and efficiency of the transport cycle, particularly in the presence of leak pathways (37). Our free-exchange model relies on both TPP^+/H^+ binding competition and the thermodynamic and kinetic asymmetry introduced by the PMF to drive productive transport by EmrE. This has an interesting parallel to the finding that the PMF controls the rate of chemiosmotically driven LacY proton/sugar symport (38).

Exactly how a membrane potential will affect the free-exchange model remains unclear. A membrane potential can affect any process involving movement of charge through the potential (39). This includes: (i) flux of charged substrates, (ii) binding and release of charged substrates if they become occluded from water, (iii) alternating access of a charged binding pocket, and (iv) structural rearrangements that modify solvent exposure of other charged areas of the protein. In the case of EmrE, all four of these are possible: all substrates are charged, the binding pocket contains two glutamates (1–3, 22), and is thus negatively charged unless protons or cationic substrates are bound, and finally there are other charged residues in the solvent-exposed loops of EmrE, which have been implicated in the transport mechanism (18). The impact of each of these components on the transport mechanism is still not well-understood and will likely vary for each transporter (25, 40, 41). Thus, we have not included a membrane potential in our current kinetic simulations of the free-exchange model.

The free-exchange model based on the NMR-detected states and transitions of EmrE predicts that EmrE should leak protons. However, we do not experimentally detect leak in any liposomal assays: protons do not rapidly flow into or out of EmrE proteoliposomes when a membrane potential is generated, and the transport processes saturate without fully dissipating the membrane potential or ΔpH in all transport assays. It is important to remember that high-resolution kinetic and structural data are almost always obtained from studies of membrane proteins solubilized in membrane mimetic environments. These data can provide great insight into the detailed molecular mechanisms. However, they must be carefully interpreted because they are not obtained in the asymmetric environment created by the proton motive force, which is necessary to drive active transport. In contrast, transport assays provide information on the effect of asymmetric gradients on net transport, but these assays cannot be performed under the solubilized protein conditions used for structural studies. Thus, when building a model from a combination of structural and transport data, it is important to remember the variety of conditions used to collect disparate datasets. Our results highlight the need to develop structural biology tools capable of experimentally determining the effect of a PMF on protein structure and dynamics.

Our transport assays confirm that EmrE does not have a single 2:1 H^+/drug^+ antiport stoichiometry, but additional experiments will be needed to understand why EmrE does not leak protons as predicted by the NMR data, determine the transport stoichiometry as a function of different conditions to understand how ΔpH and $\Delta\psi$ affect the coupling stoichiometry, and to revise the free-exchange model.

Biological Implications of the Free-Exchange Mechanism. The free-exchange model has an elegant simplicity of its own, placing no structural or dynamic constraints upon EmrE. This is functionally relevant because the highly dynamic nature of EmrE is important for its promiscuous multidrug recognition (13–15). Our model suggests that simultaneous drug and proton binding may even be advantageous, speeding up the release of tight binding

substrates (*SI Appendix, Fig. S7*) that would otherwise compromise EmrE's ability to rapidly pump toxic molecules out of *E. coli*. The free-exchange model allows for extreme adaptability in a minimalistic protein, sacrificing coupling efficiency for increased transport speed. Interestingly, reduced coupling efficiency was previously proposed as a necessary compromise for multisubstrate specificity (42, 43), and reduced efficiency may be an acceptable trade-off in the context of a bacterial cell that continuously regenerates the PMF, similar to futile ATP hydrolysis by P-glycoprotein (44, 45).

The possibility of multiple transport pathways for a single substrate also offers mechanistic flexibility for promiscuous transport of diverse substrates with different charge (+1 or +2) and binding affinities (which vary over five orders-of-magnitude for known EmrE substrates) (26). In the free-exchange model, partitioning between transport pathways depends on the relative rates of binding and release of each substrate to/from EmrE in the specific environment (pH, substrate concentration) on each side of the membrane, as well as the rate of alternating access in each drug- or proton-bound state. Different transport pathways may be faster for different substrates or different environmental conditions, resulting in different coupling stoichiometries. The coupling stoichiometry will determine the net charge movement and how strongly transport will be driven by ΔpH or $\Delta\psi$. This may be advantageous, since changes in environmental pH affect the relative contribution of ΔpH and $\Delta\psi$ to the PMF. Thus, mechanistic flexibility provides favorable transport mechanisms for a broader set of substrates and in a diverse set of conditions.

Pure-exchange models are appealing due to their apparent simplicity, tight coupling, and stoichiometric antiport. However, even a simple shared-carrier model, which allows for alternating access of the apo transporter, will give rise to coupled antiport (9). The data presented here take this one step further, demonstrating that the promiscuous transporter, EmrE, can move both substrates across the membrane simultaneously. This has been assumed to be behavior indicative of a symporter, and not possible for an antiporter. The flexibility inherent in our free-exchange model accommodates the observed ease of converting SMR transporters from antiporters to symporters (46–48), and suggests that this mechanism may be common across the SMR family. This kinetically controlled model relies upon the relative rates of substrate binding and alternating access to determine partitioning among possible transport pathways and net coupling stoichiometries. In fact, our data, while not conclusive, suggest that under the proper conditions, EmrE may favor symport rather than antiport of the same substrate. Since the transported substrate determines the rate of alternating access (26), it may be possible to design novel substrates which could favor SMR symport rather than antiport under physiological conditions. Such a compound would be actively imported by *E. coli*, rather than effluxed. The pH-dependent stoichiometry of LacY proton/sugar symport (49), as well as the variability in coupling efficiency for CLC exchangers, PepT_{ST} and MdfA, provide precedent for the idea that substrate identity could be a significant factor in defining the coupling stoichiometry.

In fact, a single transporter acting as both a symporter and antiporter of different substrates has been reported for W63G-EmrE (46). W63G-EmrE performs proton-coupled antiport of erythromycin but symport of bis-Tris-propane in vitro and in vivo, conferring resistance to erythromycin but performing concentrative uptake of bis-Tris-propane into *E. coli* to toxic levels (46). This unusual phenotype cannot be explained by the classic models of proton-coupled transport, which place mutually exclusive requirements on alternating access of the drug-free transporter (Fig. 1). However, the behavior of W63G-EmrE is readily explained with our kinetically driven free-exchange model. Negative linkage between proton and erythromycin binding will favor independent binding of the two substrates and antiport via a shared-carrier model. On the other hand, positive

linkage between bis-Tris-propane and proton binding will favor simultaneous drug and proton binding and symport. In our kinetic simulations, switching the negative linkage between drug and proton binding observed for WT EmrE to positive linkage by altering both the proton and drug on- and off-rates can switch WT EmrE from an antiporter to a (relatively inefficient) symporter (Fig. 5). A similar process could be occurring in our negative inside transport assay: the negative inside membrane potential along with the Eth⁺ gradient may produce conditions that favor simultaneous Eth⁺ and H⁺ binding and symport. Future experiments will be needed to more thoroughly test whether robust symport can be achieved. This will likely vary with each SMR because the interplay between the relative rates of all of the microscopic steps will be important for the efficiency and efficacy of either antiport or symport.

In an era when antibiotic resistance and drug-delivery pose a serious challenge, our results suggest a novel strategy. If an MDR efflux pump can indeed function as both a symporter and an antiporter, and this balance can be shifted by properties of the transported substrate, perhaps it can be subverted to drive drugs into bacteria, providing a new route for drug delivery. Despite decades of study, EmrE continues to reveal its surprising complexity and expand our understanding of basic transport mechanisms and multidrug efflux.

Methods

EmrE was expressed in *E. coli* BL21(DE3), purified, and reconstituted into proteoliposomes and bicelles as described previously (5, 50). NMR samples contained 0.5–1.0 mM EmrE monomer in DLPC/DHPC ($q = 0.33$) isotropic bicelles in 20 mM acetate, 100 mM MOPS, 100 mM bicine. All NMR spectra were collected on a 700 MHz Varian Inova spectrometer equipped with a room-temperature probe, processed with NMRPipe (51), and analyzed in CcpNmr analysis (52). The pH was measured at the experimental temperature just before acquiring ¹H-¹⁵N BEST-TROSY-HSQC spectra (53, 54). TROSY-selected ZZ exchange spectra (21) were acquired and analyzed as described previously, with error determined by jackknife analysis of individual residue fits (5, 55).

Proton release upon TPP⁺ addition to EmrE in isotropic bicelles was monitored in real time as in ref. 10 and quantitated with a known addition of NaOH. Assays were performed in 20 mM NaCl, pH 7 with 3, 5, and 10 nmol EmrE in triplicate and 0.9 mM TPP⁺ was added. ITC experiments were performed as described previously (26). Briefly, 5 mM TPP⁺ was titrated into 835 μM EmrE in isotropic bicelles in a TA Instruments Low Volume Nano calorimeter at 45 °C. The number of protons released was determined using the method of ref. 20. Buffer was 20 mM buffer (cacodylate, Mes, or piperazine) and 20 mM NaCl.

The kinetic simulation of EmrE transport (Fig. 6B and *SI Appendix, Table S2*) was run in Berkeley Madonna (v9.0; Kagi shareware) using the code in *SI Appendix, Appendix 1*. A Gillespie simulation using Octave (24) was performed using the code in *SI Appendix, Appendix 2*.

Proton leak was monitored by diluting EmrE proteoliposomes (50 mM sodium phosphate, 100 mM KCl, pH 7) into weakly buffered outside buffer (75 μM Phenol Red, 99 mM NaCl, 1 mM KCl, pH 7) to a final EmrE concentration of 0.8 μM . Valinomycin (1 $\mu\text{g}/\text{mL}$ final) was added to create a membrane potential (negative inside). FCCP (1 $\mu\text{g}/\text{mL}$ final) was added as a positive control. At the end of the assay, 10 nmol HCl was added.

For proton transport assays, EmrE was reconstituted into proton-tight liposomes with EmrE:lipid mole ratios between 1:400 and 1:1,000. For ΔpH -driven assays, the interior was strongly buffered pH 6 and the exterior was weakly buffered pH 8 with high potassium on both sides. For $\Delta\psi$ -driven assays, the interior was strongly buffered pH 7.5 with either 1 mM KCl and 99 mM NaCl (for positive inside liposomes) or 100 mM KCl (for negative inside liposomes). The exterior was weakly buffered at pH 7.5, and either 100 mM KCl (for positive inside liposomes) or 1 mM KCl and 99 mM NaCl (for negative inside liposomes). External pH was quantitated with addition of known quantities of NaOH or HCl and recorded with a microelectrode in real-time. Valinomycin (1 $\mu\text{g}/\text{mL}$) or protonophore (1 $\mu\text{g}/\text{mL}$) were added as indicated. EmrE was inactivated with N,N'-dicyclohexylcarbodiimide (DCCD). Full experimental details can be found in *SI Appendix*.

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