Perplexing new insight into the dynamics of the EmrE transporter

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The small multidrug resistance (SMR) protein EmrE helps to protect *Escherichia coli* against the toxic effects of small drug-like molecules that reach the cytosol. EmrE is thought to act by anti-porting bulky aromatic ternary and quaternary cations such as tetraphenylphosphonium$^+$ (TPP^+) from the cytosol into the periplasm against their concentration gradient, as driven by the coupled import of two protons from the periplasm into the cytosol. EmrE has several experimentally and computationally attractive features that have led to its intense study. It is small, with only 110 amino acids, it is readily overexpressed and purified, and it is tolerant; it retains native structure and functionality, even in lipid-free micelle solutions. EmrE has been extensively studied over the past 20 years using an array of methods ranging from molecular genetics to biophysics and structural biology (Bay et al., 2008; Schuldiner, 2009). As is often the case in mechanistic bioscience, the more we learn about EmrE the more we realize how little we understand. In this tradition and in this issue, Morrison et al. present nuclear magnetic resonance (NMR) spectroscopic data that provide unprecedented insight into EmrE and raise new questions.

EmrE and related SMR proteins were among the first multi-span helical membrane proteins to be studied by NMR methods. Early studies of the protein in organic solvent mixtures revealed that it retains its helical secondary structure under such conditions, but is bereft of stable tertiary structure (Schwaiger et al., 1998). Girvin and coworkers established that high quality solution NMR spectra can be obtained for SMR proteins in both detergent micelles and bicelles, conditions in which EmrE retains its native structure and functionality (Krueger-Koplin et al., 2004; Poget et al., 2010). Others showed that it was also possible to use solid-state NMR to make useful measurements on the protein in lipid vesicles and large bicelles (Glaubitz et al., 2000; Agarwal et al., 2007; Lehner et al., 2008; Gayen et al., 2013; Mörs et al., 2013; Ong et al., 2013; Banigan et al., 2015). In 2012, the Henzler-Wildman group presented solution NMR studies of the EmrE–TPP^+ complex that resolved pressing controversies regarding the structure and mechanism of action of this antiporter (Morrison et al., 2012). EmrE had long been known to function as a homodimer, but there was much debate (Schuldiner, 2009) as to whether it functions as a parallel dimer with both subunits sharing the same orientation in the membrane, or whether it functions as an antiparallel dimer that is asymmetric in the sense that its two subunits not only have opposition membrane orientations ("dual topology") but also different conformations. This latter possibility was consistent with the then-available medium-resolution crystal structure (Chen et al., 2007). Solution NMR studies in bicelles by Henzler-Wildman and colleagues revealed not only that EmrE functions as an asymmetric antiparallel dimer, but that the two subunit conformations interconvert when the antiporter switches from its open-in state to its open-out state (Morrison et al., 2012). This was confirmed by later solid-state NMR results (Gayen et al., 2013) and supports the notion that the EmrE crystal structure (Chen et al., 2007) reflects the native structure, albeit at modest resolution. A recent biochemical study established that the dual topology of the asymmetric dimer appears to be cotranslationally established by the ribosome and translocon (Woodall et al., 2015), a result that may shed light on the mechanisms of assembly of other known dual topology membrane proteins (Rapp et al., 2006; Duran and Meiler, 2013). The new paper by Morrison et al. (2015) presents NMR results that complement and extend previous studies of EmrE by documenting two properties that need to be incorporated into our thinking about how this protein works.

The initial focus of the study by Morrison et al. is on the critical pair of Glu14 residues located in the transmembrane domain of both subunits. It is thought that the carboxyl side chains of these residues serve as the carrier sites for the two protons that are imported into the cytosol to energetically drive the EmrE transport cycle, operating according to alternating access model (Schuldiner, 2009). Upon transition to the open-in state, the imported protons dissociate into the cytosol. The two deeply buried Glu14 carboxylates are thought to then play a crucial role in the recognition and binding of transported substrates such as TPP^+. Protonation

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of each Glu14 is directly competitive with substrate binding; a single substrate molecule can bind only when both Glu14 residues are deprotonated. Carefully acquired data from the Schuldiner group was interpreted previously to indicate that both Glu14 residues have the same pK_a. In one study, the pH dependency of proton release from EmrE was used to estimate that this pK_a is 8.4 (Soskine et al., 2004). Later, Trp fluorescence was used to measure the pH dependency of k_{on} for TPP^+ binding, leading to the conclusion that the shared pK_a is 7.3 (Adam et al., 2007).

In the present work, a series of NMR and mutagenesis experiments was performed on EmrE solubilized in membrane-mimicking bicelles that convincingly establishes that the two Glu14 residues present in a single homodimer have very different pK_a values, one in the 6.8–7.0 range and the other in the 8.2–8.5 range. Therefore, the structural nonequivalence of these two residues is reflected in dramatically different acid-base properties. Both pK_a values are, of course, elevated by 3–4 units relative to pK_a values for water-exposed Glu residues, reflecting the highly apolar environments of these sites in the EmrE dimer.

The cytosol of *E. coli* is maintained in the pH 7.6–7.8 range (Kashket, 1985), in between the two Glu14 pK_a values. This indicates that when the EmrE is in the substrate binding–competent inside-open state, one of the Glu14 residues will be mostly protonated, whereas the other will be mostly deprotonated. This suggests that substrates will bind to the relatively rare (at pH 7.6–7.8) form of EmrE where both Glu14 residues are deprotonated and/or bind (more weakly) to the singly deprotonated state, at which point the pK_a of the other Glu14 proton be will shifted to a lower value followed by dissociation of that proton and completion of high affinity substrate binding. It is noted that the K_d for substrate binding to the transporter varies little (Adam et al., 2007) between the cytosolic pH of 7.6–7.8 and values near 9.0, where both Glu residues will be mostly deprotonated.

The pH of the bacterial periplasm is usually reduced compared with the cytosol, depending mostly on the pH of the physiological milieu. This means the Glu14 with the pK_a of ∼8.3 will usually be rapidly and completely protonated in the open-out state. The K_d for TPP^+ binding to EmrE increases sharply as the pH is reduced below 7.2, with this trend primarily reflecting increases in k_{off} (Adam et al., 2007). This suggests that both the rate and thermodynamics of substrate dissociation from EmrE into the periplasm is critically dependent on the protonation state of the second (pK_a of 6.8–7.0) Glu14. In this regard, it is interesting to note that k_{off} for TPP^+ at pH 6.9 is $\sim 0.5\ s^{-1}$, whereas the rate of open-in and open-out exchange of the EmrE–TPP^+ complex is roughly an order of magnitude faster (Adam et al., 2007; Cho et al., 2014). This suggests that at pH values at and above the pK_a of the more acidic of the two Glu14 residues, conformational switching of the EmrE–substrate complex only rarely leads to actual transport from the cytosol and release into the periplasm, at least when that substrate is TPP^+. Is this not surprising?

A second and provocative observation from the study by Morrison et al. (2015) extends recent NMR results from the Traaseth laboratory (Cho et al., 2014). It has long been known that EmrE is structurally plastic, enabling recognition and binding of a structurally wide range of hydrophobic cation substrates (Ubarretxena-Belandia et al., 2003; Fleishman et al., 2006; Korkhov and Tate, 2008). However, NMR results showed that in addition to this conformational plasticity within a structural state, substrate-free EmrE undergoes rapid ($300\ s^{-1}$ at pH 6.9 and 37°C) constitutive conformational switching of subunit conformations, transitions that correspond to exchange between drug-free open-in and empty open-out structures in bilayered vesicles or cells (Cho et al., 2014). Although constitutive conformational switching between different functionally relevant states is a time-honored concept that applies, for example, to many signaling proteins, the realization that an antiporter rapidly and constitutively interconverts so rapidly between open-in and open-out states is remarkable. Henzler-Wildman and colleagues present data in the present paper that makes this observation even more perplexing. They showed that even at pH 8.8, where 80% of the transporter has both Glu14 residues in their carboxylate forms and only 1% have both Glu14 residues in their acid form, the rate of conformational switching is $50\ s^{-1}$, still much faster than the transport rate observed for most EmrE substrates (compare Morrison and Henzler-Wildman, 2014). Does this mean that the EmrE dimer with both Glu14 residues deprotonated can rapidly and spontaneously execute the conformational switch required for transport? Or, could it be that only the rare (at pH 8.8) fully protonated form can execute the subunit conformational swap, doing so at a rate of $100 \times 50\ s^{-1}$, with rapid (compared with the switch) equilibration of protons between the various conjugate acid/base forms present in the total population of EmrE molecules?

Even to a non-expert in transport and bioenergetics such as the author of this commentary, the results of these studies suggest that many moons will pass before the nature of the EmrE transport cycle can be considered to be well understood in terms of its mechanism, energetics, structure, and dynamics. Indeed, one wonders if even the notion that EmrE harnesses proton import to drive substrate export into the periplasm against a concentration gradient might not be in question. There is evidence that the transport of substrates by EmrE and other SMR proteins from the cytosol to the periplasm is coupled to active transport of these same

substrates out of the periplasm and into the environment by an AcrAB-TolC multidrug efflux transport system, which spans both membranes (Tal and Schuldiner, 2009). Could it be that EmrE sometimes operates to export compounds to the periplasm under conditions in which it actually does not have to work against a concentration gradient? If so, then it suggests that the role(s) for protonation of the Glu14 side chains in the function of EmrE, although unquestionably crucial, may at least sometimes be other than to drive uphill transport.

None should weep because there are no interesting questions left to address for the littlest of membrane transporters.

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