Chapter 10
What Can a Living Fossil Tell Us About Evolution and Mechanism of Ion-Coupled Transporters: The Story of Small Multidrug Transporters

Shimon Schuldiner

Abstract Small Multidrug Resistance (SMR) transporters are small homo- or hetero-dimers that confer resistance to multiple toxic compounds by exchanging substrate with protons. They reside in the inner membrane of bacteria and in halophilic and methanogenic archaea and because many of their substrates are routinely used as antibiotics and antiseptics, they have been associated with the phenomenon of multidrug resistance. EmrE, the most studied SMR member, has presented biochemists with unusual surprises regarding its topology and raised an interesting controversy since structural information was in an apparent conflict with biochemical data. One of the reasons for the controversy was the assumption that, to ensure proper function, membrane proteins must be inserted by a mechanism that warrants a unique topology. As it turns out, EmrE and other SMR transporters display a remarkable plasticity regarding topology in the membrane, interaction between subunits, and interaction with substrates. This plasticity implies a high evolvability of these proteins and, as a consequence, a lack of commitment that facilitates acquisition of new functions and topologies. Because of this high evolvability, we suggest that SMRs are living fossils at an evolutionary junction. Study of their properties provides a wonderful glimpse at the evolution and mechanism of ion-coupled transporters.

Keywords Antiporter • Multidrug transporters • Parallel and antiparallel topology • Proton release • Transient kinetics • Tryptophan fluorescence • Uncoupled mutants

S. Schuldiner (✉)
Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel
e-mail: shimon.schuldiner@huji.ac.il

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10.1 Living Fossils and Modern Mechanisms

Living fossil is an informal term for any living species or organism which appears to be the same as a species otherwise only known from fossils and which has no close living relatives. These species have all survived major extinction events and generally retain low taxonomic diversities. I suggest that EmrE and SMR proteins resemble, in a way, living fossils that are widespread only in bacteria and in very few groups of archaea (Schuldiner 2009; Kolbusz et al. 2013; Bay and Turner 2009). Because of their high evolvability we suggest that SMRs are at an evolutionary junction. Evolvability can be seen also as lack of commitment: EmrE and other SMRs are not fully committed to a certain substrate and can easily change specificity (Brill et al. 2012) and even mode of coupling (Brill et al. 2012; Adam et al. 2007); they are not committed to a certain topology (Nasie et al. 2010) and not even committed to a certain partner to form the oligomer (Nasie et al. 2010; Schuldiner 2012).

In this chapter, I will concentrate on what we have learned from the study of EmrE and I will compare the mechanism proposed for EmrE to that in other H^+-coupled antiporters. EmrE is a small (110 residues) SMR transporter from *Escherichia coli* that functions as a dimer and extrudes positively charged drugs in exchange for two protons, thus rendering bacteria resistant to a variety of toxic compounds. Study of this small, 110-residue multidrug transporter from *E. coli* has provided valuable information for the understanding of the coupling mechanism of the ion-coupled transporter family (Yerushalmi and Schuldiner 2000a, c; Soskine et al. 2004; Schuldiner 2007, 2009; Adam et al. 2007). The simplicity of the coupling mechanism in EmrE allows for the remarkable flexibility in the structure of the protein that will be discussed below.

10.1.1 Competition as a Way of Life for H^+-Coupled Antiporters

H^+-coupled antiporters are ubiquitous proteins that utilize proton electrochemical gradients generated by primary pumps. As a result, these proteins remove their substrates from the cytoplasm into acidic intracellular compartments or out to the medium. Well-known examples of this type of transporters are the multidrug transporters, vesicular neurotransmitter transporters, organic anion, cation transporters, and Na^+/H^+ antiporters.

H^+-coupled antiporters have been proposed to couple transport by utilizing a sequential binding and translocation mechanism, through which the substrate must be released prior to binding and translocation of the counter-transported ion (Fig. 10.1). Such a mechanism anticipates two major conformations of the transporter, facing alternatively each side of the membrane (C_o and C_i), which can interconvert only when one of the substrates is bound. Furthermore, binding of

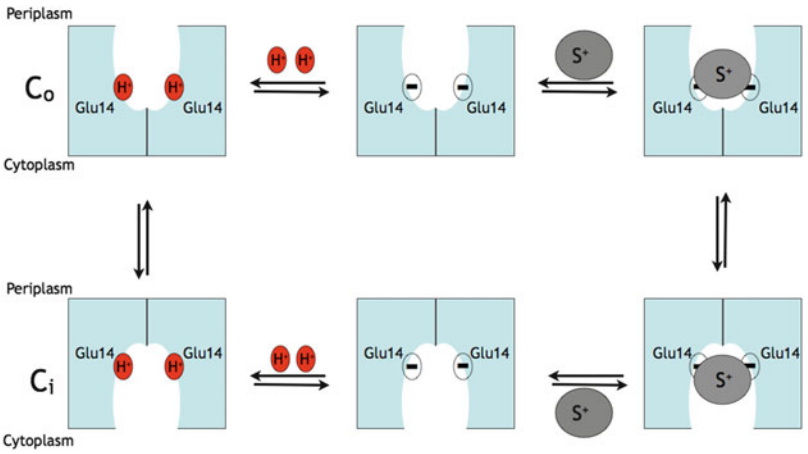

Fig. 10.1 *Alternating access model of EmrE transport*. Each state in the transport cycle is only open to one side of the membrane, and the two states only interconvert when either two protons (*small red circles*) or one substrate molecule (*grey circle*) are bound. Glu14 is the only membrane embedded carboxyl (*small circles* with *negative sign*) in each one of the protomers in the functional dimer

the counter substrates together is prohibited. This implies a competition between the two substrates that maybe direct as in the case of EmrE or indirect, in other examples that are discussed in detail below.

10.1.1.1 The Unbearable Simplicity of the Coupling Mechanism in EmrE

EmrE provides a unique experimental paradigm not only because of its size and stability but because, under proper conditions, the detergent solubilized protein binds substrate and releases protons in a mode that reflects with high fidelity its catalytic activity in the membrane. This property has enabled a detailed study of the molecular basis of substrate recognition and the coupling between protons and substrate (Yerushalmi and Schuldiner 2000a, b, c; Yerushalmi et al. 2001; Soskine et al. 2004; Gutman et al. 2003; Weinglass et al. 2005). EmrE contains eight charged residues, seven of them located in the hydrophilic loops and only one membrane-embedded charged residue, Glu14, which is also conserved in more than 200 homologous proteins in bacteria and archaea (Bay et al. 2008; Schuldiner 2009). Replacement of Glu14 in EmrE or Smr from *S. aureus* (equivalent Glu13) with Cys, Gln, His, Tyr, or Asp had a profound effect on the phenotype (Grinius and Goldberg 1994; Yerushalmi et al. 1995; Muth and Schuldiner 2000; Yerushalmi and Schuldiner 2000b). Further characterization of the mutants showed that the E14C mutation yielded a protein completely devoid of activity, while the E14D mutation was impaired in its ability to couple substrate fluxes to the proton gradient

but was able to bind substrate and transport it downhill (Yerushalmi and Schuldiner 2000b). An EmrE mutant with a single carboxyl at position 14 was constructed by replacing the two carboxyls in the loops with Cys residues. This single carboxyl mutant displays properties similar to those of the wild-type protein, indicating that out of the three acidic residues of the protein, the only essential one is Glu14 (Yerushalmi et al. 2001).

10.1.1.2 A Dual Role for Glu14

The pH dependence of substrate binding and the properties of the Asp replacement at this position reveal the dual role of Glu14 in catalysis: it plays a role in both substrate and H^+ binding, and the occupancy of the site is mutually exclusive. Substrate binding to either the wild-type protein or the single carboxyl mutant increases dramatically with increasing pH suggesting that deprotonation of Glu14 is required [Fig 10.2 and Muth and Schuldiner (2000), Yerushalmi and Schuldiner (2000a), Yerushalmi and Schuldiner (2000c)]. Conversely, substrate induces proton release, and both reactions (substrate binding and proton release) have been observed directly in the detergent solubilized preparation of EmrE (Soskine et al. 2004).

In addition to the steady state measurements, the use of transient kinetics allowed us to break down this multistep process to its individual steps (Adam et al. 2007). A powerful tool was provided by the study of the fluorescent properties of EmrE. The fluorescence of Trp63 in EmrE reflects the occupancy of the binding site in the protein: the highest fluorescence is observed when the protein is fully deprotonated; the protonated protein displays a lower fluorescence, and the substrate bound protein displays the lowest [Fig. 1 in Adam et al. (2007)]. Interestingly, the environment to which Trp63 is exposed, as reflected by the wavelength of its fluorescence peak, does not change significantly at the three different states suggesting that Trp63 does not go through large conformational changes that expose it to different environments. A possible reason for the different fluorescence levels could be due to quenching or energy transfer to the substrate or to other amino acids not yet identified.

These findings led us to study the transient kinetics of substrate binding and proton release using spectroscopic techniques: the fluorescence of either the wild-type protein or one with a single Trp at position 63 was used to measure binding rates (Adam et al. 2007). The maximal rates of substrate binding of about $2 \times 10^7\ M^{-1}\ s^{-1}$ are relatively close to typical values in diffusion limited processes, implying that binding does not necessitate major conformational changes of the protein. The k_{on} values for binding to the single Trp63 mutant in the pH range measured are essentially identical to the k_{on} values for binding to the wild-type protein. These findings further validate the use of the mutant to study the mechanism of catalysis. Moreover, the results suggest that Trp63 is the one responsible for most of the fluorescence changes in the wild-type protein. The changes in

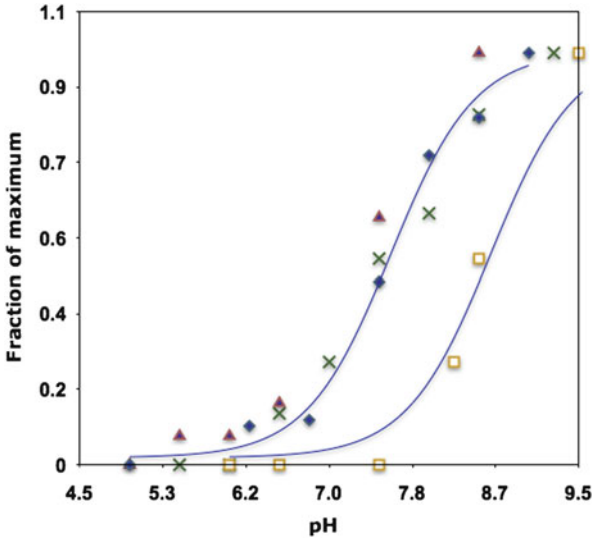

Fig. 10.2 *pH dependence of substrate binding and transport in EmrE*. Rates (*multiplication symbol*) and steady state (*filled diamond*) levels of TPP^+ binding to detergent solubilized EmrE increase with pH in a manner practically identical to the increase of downhill rates of efflux of methyl viologen (*filled triangles*) from proteoliposomes reconstituted with EmrE. A pH gradient of 2 units was imposed to drive methyl viologen accumulation into proteoliposomes. In these experiments the rate increases with increasing pH, but there is a shift of approximately 1 pH unit to the alkaline side (squares). Data from references Adam et al. (2007), Yerushalmi and Schuldiner (2000b) and Muth and Schuldiner (2000)

fluorescence of Trp63 are extremely large, and under optimal conditions most of the fluorescence is quenched either by protons or by substrate.

10.1.1.3 The Proposed Mechanism

Taken as a whole, the results support a binding mechanism where binding of either protons or substrate requires dissociation of the other. The suggested reaction path implies that first $EH_2 + S$ dissociate to $E + S + 2H^+$, and it rapidly leads to the formation of $ES + 2H^+$. The model assumes, as a first approximation, no binding to either the singly or doubly protonated protein and that both protons have a single or very similar pK_a [summarized in Adam et al. (2007)]. The experimental support for this model is based on the following findings:

1. Stoichiometry of substrate-induced proton release as determined from steady state measurements with detergent solubilized purified EmrE is $2H^+$/functional unit (dimer) (Soskine et al. 2004). This finding is supported also by stoichiometry of the whole transport process as measured in the intact *E. coli* cell: $2H^+$/substrate (Rotem and Schuldiner 2004).

2. In steady state measurements the pH dependence of the substrate-induced release of protons supports the contention that the pK_A must be well above 7 (Soskine et al. 2004). This contention is supported also by the pH dependence of the Trp63 fluorescence (Adam et al. 2007).
3. Although EmrE releases two protons upon substrate binding, the substrate induced proton release is a first order reaction, supporting the contention that the two protons display identical or very similar pK_A values.
4. Substrate binding shows a time course fit with a single exponential.

When results from the pre-steady state measurements are fitted to this simple model, the pre-equilibrium pK_A is predicted to be ~7.3 for the wild-type carboxyl at position 14 (Glu14) and ~5.8 for the aspartate replacement (Asp14). The pK_A values from this fit are lower than the ones estimated from steady state measurements (8.3–8.5 and 6.7, respectively) (Soskine et al. 2004). The reason for this apparent discrepancy is not fully understood. It may be due to technical limitations: a lower accuracy of the steady state measurements or for the need to obtain a model that better describes the reaction and predicts other pK_As. It may also reflect the fact that in the described experiments we are measuring two different parameters: substrate-induced initial rates of proton release in one case and quantity of protons released in the steady state measurements.

10.1.1.4 Uncoupled Mutants Raise Interesting Questions

The mechanism suggested above necessitates the fine-tuning of the pK_A of the carboxyl or the carboxyls participating in the protonation and deprotonation reactions. This contention was experimentally demonstrated in EmrE where replacement of Glu14 with Asp results in a decrease of the pK_A of the carboxyl. The mutant protein with a lower pK_A is mostly deprotonated at physiological pH, so it binds substrate but cannot couple the substrate flux to the proton gradient (Yerushalmi and Schuldiner 2000b; Soskine et al. 2004).

The behavior of this mutant raises a general question regarding antiporter mechanisms. In a classical scheme of an antiporter cycle, it is assumed that the transition of the "unloaded" transporter between the conformations facing the opposite sides of a membrane is not allowed (Fig. 10.1). Should such a transition be permitted, the coupling between the two fluxes would be compromised. A single conservative mutation of Glu14 to Asp transforms an antiporter into a uniporter suggesting that, in the mutant, this transition is permitted. It is possible that the mutation has an effect on the transporter that modifies the likelihood of this step to occur. Another more interesting possibility is that the transition is always allowed, even in the wild-type EmrE. The reason that it is not detectable is that the concentration of the deprotonated form of the wild-type transporter is, under most conditions, extremely low. In the E14D mutant that displays a lower pK_A, a large fraction of the transporter is deprotonated and can then interconvert freely between C_o and C_i conformation without the protonation reaction.

10.1.1.5 Validation of the Binding Measurements

Although they provide invaluable information, the measurements of the binding and release reaction with a detergent solubilized protein need validation with transport experiments performed with the protein in the membrane. Downhill efflux and exchange experiments were performed with proteoliposomes reconstituted with purified EmrE (Yerushalmi and Schuldiner 2000b). Downhill transport of substrate involves all the steps described in Fig. 10.1 but is driven by the substrate electrochemical gradient and does not necessitate a gradient of H^+ ions, i.e., the pH inside and outside the proteoliposome are the same. When the rate of Methyl Viologen efflux was measured at various pH values, it was found that the increase in the rate with an increase in pH was identical to that of the binding reaction measured in detergent [Fig. 10.2 and see also Yerushalmi and Schuldiner (2000b)]. In exchange experiments, a saturating concentration of unlabeled substrate is included in the outside solution and the transporter thus interconverts between the inside and the outside conformations in the "substrate-loaded" mode. Also in these experiments the pH dependence of the reactions was similar to that of the binding reaction (Yerushalmi and Schuldiner 2000b). When active uptake was measured, a pH gradient of 2 units was imposed to drive the accumulation. In these experiments the rate increases with increasing pH, but there is a shift of approximately 1 pH unit to the alkaline side suggesting that Glu14 is now "sensing" the average between the internal and external pH of the proteoliposome [Fig. 10.2 and see also Yerushalmi and Schuldiner (2000b)]. This "averaging" may be the result of the alternate exposure of the carboxyl to the acidic and alkaline face of the liposome. When similar experiments are performed with the "uncoupled" E14D mutant, the rates of efflux and exchange are practically independent of pH in the range 5–9 as expected from a protein with the essential carboxyl deprotonated at these pH values (Yerushalmi and Schuldiner 2000b).

These results validate the experiments performed with the detergent solubilized transporter and support the dual role proposed for Glu14.

10.1.1.6 Direct and Indirect Competition in Other Antiporters

As described in detail above, in the case of EmrE, the coupling mechanism is based on a direct competition of the substrate and the H^+ for the same binding site. Is there evidence for a similar mechanism for the larger modern antiporters? The answer is most likely a complex one.

NhaA provides another well-documented case of a direct competition between the two substrates (Mager et al. 2011). NhaA is a Na^+/H^+ antiporter from *E. coli* that belongs to the CPA family of monovalent cation/proton antiporters (Padan et al. 2009). It plays a central role in sodium and proton homeostasis by exchanging two H^+ with one Na^+ ion (Taglicht et al. 1993). Electrophysiological measurement of the activity of NhaA was tested over a wide pH range from pH 5.0 to 9.5.

Forward and reverse transport directions were investigated at zero membrane potential using preparations with inside-out and right side-out oriented transporters with Na^+ or H^+ gradients as the driving force. Under symmetrical pH conditions with a Na^+ gradient for activation, transport exhibited highly symmetrical activity with a bell-shaped pH dependence. The pH dependence was associated with a systematic increase of the *Km* for Na^+ at acidic pH. Under symmetrical Na^+ concentration with a pH gradient for NhaA activation, an unexpected novel characteristic of the antiporter was revealed; rather than being downregulated, it remained active even at a pH as low as 5 (Mager et al. 2011). This classical competition seems to be at a common binding site, as suggested by biochemical and structural evidence that support a mechanism whereby two Asp residues, Asp163 and Asp164, provide the site for both H^+ and Na^+ (Arkin et al. 2007; Padan et al. 2009).

On the other hand, at least in two cases, the counter-transported substrates seem to bind at different sites without compromising the competitive nature of their binding. Thus, in the case of MdfA, a proton/drug antiporter from *E. coli* that belongs to the MFS family, genetic and biochemical evidence support the contention that two distinct binding sites may exist for substrates and protons (Fluman et al. 2012). PfMATE, a H^+/drug antiporter from *Pyrococcus furiosus* that belongs to the MATE family, was recently crystallized (Tanaka et al. 2013). Structural and biochemical evidence support the contention that the binding sites for H^+ and substrates do not overlap.

An extreme example of "long-distance" competition is provided by the AcrAB-TolC complex from *E. coli*, a large tripartite complex that belongs to the RND family and provides the major intrinsic resistance of these cells. It actively removes a large variety of drugs from the periplasm by a mechanism that utilizes the proton electrochemical gradient across the cytoplasmic membrane. In the case of the AcrAB-TolC complex, there is a complete spatial separation of the sites: the proton binding site is in the membrane domain while the substrate binding site is in the periplasmic portion of this large complex (Seeger et al. 2009). The conformational changes occurring upon protonation and deprotonation of carboxylic residues in the membrane domain are coupled to conformational changes in distant domains where the substrate transport takes place (Seeger et al. 2009).

A notable, and maybe not unique, exception to the mechanism depicted in Fig. 10.1 is presented by the ClC Cl^-/H^+ antiporter (Miller and Nguitragool 2009). Antiporters bind their substrates alternately in the two different states and shuttle them across the membrane with ping-pong like conformational changes according to the alternating access model. In contrast, ClC is able to bind both its substrates simultaneously, and, importantly, proton movement along its pathway seems to be possible only when the central anion binding site is occupied by Cl^- (Miller and Nguitragool 2009).

Noteworthy, in three of the cases described above, NhaA, MdfA, and AcrAB-TolC complex, the pK_A of the important residues has been estimated to be at around 7.5 (Seeger et al. 2009; Fluman et al. 2012; Arkin et al. 2007; Mager et al. 2011), a value well within the range of the intracellular pH of *E. coli* cells (Padan

et al. 1981). Thus, it seems that regardless of their specific structures or mechanisms, the transporters have evolved so that they are exquisitely tuned to function at the very constant cytoplasmic pH maintained by *E. coli* cells.

10.1.2 Mechanism and Structure

Much has been said about the structure and topology of EmrE [reviewed in Schuldiner (2012), see also Chap. 11]. The available models of the structure are derived from low-resolution 2D and 3D-crystals (Fleishman et al. 2006; Chen et al. 2007). To explain the quasi-symmetry observed in the 3D structure of EmrE acquired by electron cryo-microscopy (cryo-EM), Ubarretxena-Belandia et al suggested the possibility of an antiparallel topology of the protomers in the EmrE homodimer (Fig. 10.3) (Ubarretxena-Belandia et al. 2003). Very soon thereafter, a model derived from a low-resolution 3D-crystal structure supported this suggestion (Pornillos et al. 2005). Even though the structure was withdrawn (Chang et al. 2006), it stimulated investigators in the field to pursue this unique avenue. Fleishman et al. used the symmetry relationship mentioned above, combined with sequence conservation data, to assign the transmembrane segments in EmrE to the densities seen in the cryo-EM structure. The C-α model of the transmembrane region was constructed so that the helices of one protomer have the topology opposite to the ones of the other protomer (Fig. 10.3, antiparallel) (Fleishman et al. 2006). A recalculated model based on the 3D-crystals is similar to the model suggested from the electron microscopy data (Chen et al. 2007). The crystals used to derive the C-α model X-ray structure were obtained with protein solubilized with detergents that inhibit activity by disrupting the oligomer (Chen et al. 2007; Soskine et al. 2006). The protomers thus obtained arrange in the crystal in a conformation that minimizes the energy needed for crystal formation and do not necessarily reflect the topology in the membrane.

In addition, genetic experiments were designed to support the claim for an antiparallel topology. EmrE was fused to the "topology-reporter proteins" alkaline phosphatase and green fluorescent protein, and the results showed that the topology of the EmrE fusion proteins in the membrane is sensitive to the distribution of positive charges in the protein (Rapp et al. 2006). Manipulation of the positive charges generated a set of mutants, some with N_oC_o, and others with N_iC_i apparent topology (Rapp et al. 2006, 2007). Since neither mutant conferred resistance to ethidium, the authors concluded that this was due to the modified topology. Co-expression of the inactive mutants restored the ethidium resistance phenotype to the same level as seen with wild-type EmrE (Rapp et al. 2007). The suggested interpretation of this finding was that co-expression results in the generation of a functional, antiparallel heterodimer. However, this conclusion is based solely on the contention that the N_iC_i and N_oC_o mutants are inactive. This conclusion necessitates further experimental support, because the mutants that were designed to insert into the membrane with the N_iC_i and N_oC_o topology are both functional

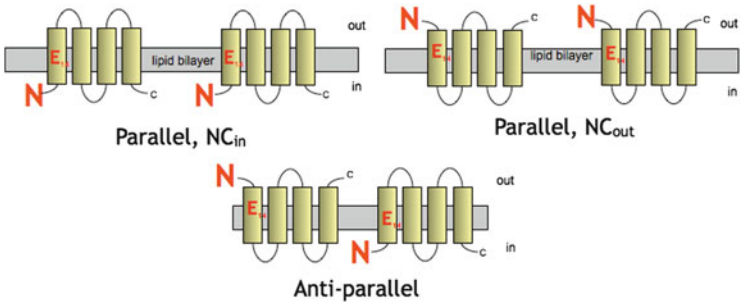

Fig. 10.3 *Three topoforms of EmrE*. The topogenic determinants in the wild type EmrE are nonexistent and therefore parallel dimers insert in random topologies: about half N_iC_i and half N_oC_o. After insertion, the interaction of the protomers will result in a parallel or antiparallel mode depending only on their relative affinities. Adapted from reference Schuldiner (2012)

and capable of removing substrate from the cell when the phenotype is assayed in cells with the proper genetic background (Nasie et al. 2010) or following growth continuously in liquid medium (McHaourab et al. 2008). The lower activity of the single mutants compared with the co-expressed ones is most likely due to impaired dimerization and not necessarily due to a different topology (Nasie et al. 2010).

10.1.2.1 Evidence Supporting Parallel Topology

To validate the above-mentioned structural models, we tested one of their most basic predictions: the existence of antiparallel dimers. All our experiments were consistent only with parallel dimers. Unique cysteines were engineered in the hydrophilic loops or at the termini, and crosslinkers that could react with residues 9–11 Å apart quantitatively cross-linked the protomers (Soskine et al. 2002). This finding is inconsistent with an antiparallel topology, since in such a case the Cys would be at least 35–40 Å apart. Since cross-linking could be amenable to artifacts, we also purified two proteins cross-linked in different positions and showed that they are fully active supporting the contention that the crosslinking experiments reflect the situation in functional dimers (Soskine et al. 2006). Generation of genetic fusions (tandems) where the C-terminus of one protomer was fused to the N-terminus of the second protomer provided a paradigm to study the topology both in vivo and in vitro. The linkers were designed such that they are very short or very hydrophilic. This ensured that both termini are on the same side of the membrane and force the dimer into a parallel topology (Steiner-Mordoch et al. 2008). All the tandems built as described were functional in vivo and after purification. Control experiments were performed to ensure that the functional unit is the dimer and not a result of interaction of dimers.

10.1.2.2 Multiple Topologies of SMRs

Any single dimeric membrane protein may, in theory, adopt three different topologies (denoted here as "topoforms") schematically shown in Fig. 10.3. Relative to each other, the two protomers can theoretically adopt either a parallel (N and C-termini of both protomers on the same side of the membrane) or anti-parallel orientation (N and C-termini of each protomer on opposite sides of the membrane). Additionally, relative to the lipid bilayer, the ensemble of parallel dimers can allegedly adopt a single topology or a dual topology where dimers are either all in a N_iC_i (or N_oC_o) orientation or a mixture of both. However, while all the potential topologies could occur what topology makes biological sense? The answer to that question is open since there are constraints such as the way the protein is inserted into the membrane during synthesis and the requirement for the dimer to be catalytically active.

One of the reasons for the controversy about the topology of EmrE was the assumption that to ensure proper function membrane proteins must be inserted in a unique topology. Indeed, it is obvious that proteins such as receptors must face the environment they are probing, channels that sense the electrical field across membranes depend on the polarity of the field and cannot be inserted randomly, pumps that derive their energy from molecules such as ATP, available only on one side of the membrane, need to have the ATP utilization machinery in the right location. However, is a unique topology a necessary prerequisite for uniporters or ion-coupled transporters?

H^+-coupled transporters couple the free energy released from downhill translocation of H^+ in response to an H^+ electrochemical gradient to drive the energetically uphill stoichiometric flux (in or out) of their substrates. As such, they function as thermodynamic nano-devices capable of transporting in either direction, the direction dictated solely by the driving gradients. There is no need to assume differences in affinities from both sides of the membrane for such a mechanism to hold [see for example, Guan and Kaback (2004)]. Therefore, H^+-coupled transporters should be essentially symmetrical in their function unless there is a specific need for rectification or regulation of their function.

To account for the observation that both parallel and antiparallel dimers are functional, we propose that this is possible because the mechanism of coupling ion and substrate transport in EmrE is so simple: two carboxyls with a properly tuned pK_A located approximately in the middle of the membrane provide the core of the coupling mechanism. This simplicity provides the robustness necessary to tolerate such a unique and unprecedented ambiguity in the interaction of the subunits and in the dimer topology relative to the membrane.

10.1.3 SMRs and Evolution of Transporters

The behavior observed with EmrE and other SMRs may represent a stage in the evolution of the topology of membrane proteins (Schuldiner 2007, 2009, 2012). The existence of homodimers and heterodimers in this family of small H^+-coupled multidrug transporters provides an exceptional paradigm to study the evolution of heterodimers and of the larger transport proteins. The heterodimers have apparently originated from gene duplication of the more ancient homodimers. After gene duplication, a relatively small number of mutations may be sufficient to convert a homodimer into a heterodimer. The large transporters may then have arisen from a series of gene fusions. The small SMRs did not survive as such during evolution and genes coding for them are found mainly in the bacterial world with a few representatives in the Archaea but none, thus far, in the eukaryotes.

We hypothesize that the appearance of heterodimers may have provided an evolutionary advantage. Thus, for example, a single mutation in an essential residue of a functional homodimer would lead to complete loss of activity. Partial activities have been observed in in vitro-produced heterodimers even when one of the protomers carries a mutation in an essential residue (Ninio et al. 2001; Sharoni et al. 2005; Rotem et al. 2001; Steiner-Mordoch et al. 2008). Thus, the above handicap is partially overcome in the heterodimers that have evolved from the primitive homodimer after gene duplication.

Another advantage for heterodimers is the potentially higher diversity that can be achieved by combinations of mutations, a diversity that may have been critical in proteins that should recognize a wide range of substrates. Yet, an additional advantage for such functional dimers is also hinted at by the ongoing controversy regarding their topology. In many of the putative heterodimers, the topological determinants are apparent and predict an antiparallel topology. In some, as judged from the same determinants, the interaction may be parallel. On the other hand, in homodimers such as EmrE, the need for topological determinants in such a small protein may be too much of a burden, a burden that can be alleviated in larger proteins where single signals may be better tolerated. When these determinants are engineered by mutagenesis, they impair activity (Rapp et al. 2007), most likely because of the diminished interaction between monomers dictated by the high density of positive charges in the loops (Nasie et al. 2010).

The next step in the evolution of transporters has been the generation of the larger ones that usually function as monomers. A structural approach has revealed that many ion-coupled transporters are found as oligomers in crystals and most likely also in the membrane. However, the functional significance of the finding is not yet evident since in many cases it has been shown that the substrate translocation pathway and the coupling gear are in the monomer (Geertsma et al. 2005; Yernool et al. 2004; Veenhoff et al. 2002; Lanyi 2004; Bamber et al. 2007; Williams et al. 1999; Fujiyoshi et al. 2002). Even though the monomers are the functional unit, in some cases, their interaction appears necessary for catalysis [e.g., in the case of the RND transporters (Eicher et al. 2009) and lactose transport

(Veenhoff et al. 2002)], stability (Herz et al. 2009), or for regulation (Perez et al. 2011).

Why did the small proteins not survive the process of evolution? Maybe it simply was an evolutionary dead end. However, it could be that the necessity to add soluble loops for regulation, targeting to specific membranes and interaction with other components of the cell, selected in favor of the larger transporters where these additions are less of a burden.

Inverted repeats have been identified in many large modern transporters and have been suggested to play important roles in the transport mechanism (Forrest and Rudnick 2009). We propose that the plasticity described here could provide an example of how inverted repeats in modern large polytopic transporters may have developed. The interaction interfaces must be appropriate for generation of a stable oligomer, and a substrate binding cavity must supply interaction points at given and fixed locations. We suggest that the promiscuity among interacting protomers reported here is tolerated also functionally because in EmrE, as in other proteins interacting with multiple substrates, the size of the binding pocket must be large enough to allow different molecules to reside in it in different orientations and to establish interactions with different sets of residues on the pocket walls (Seeger et al. 2006; Murakami et al. 2006; Godsey et al. 2002). Therefore, the minimal structure where we find the necessary elements for substrate recognition and coupling may be provided by a dimer with the protomers in either one of the possible topologies.

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