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# When biochemistry meets structural biology: the cautionary tale of EmrE

**Shimon Schuldiner**

Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

**When biochemistry meets structural biology a more complete understanding of the mechanism of biological macromolecules is usually achieved. Several high-resolution structures of ion-coupled transporters have enriched the understanding of mechanisms of substrate recognition, translocation and coupling of substrate fluxes. However, two X-ray structures of EmrE, the smallest ion-coupled multi-drug transporter, raised questions over the veracity of the structural model and represented a cautionary tale about the difficulty of determining the 3D structures of membrane proteins and the dangers of ignoring biochemical results. The 3D structures of EmrE have since been retracted because of faulty software, but the suggestion that the protomers in the dimer are in an antiparallel topological orientation sparked controversy that is still ongoing.**

## EmrE, a unique experimental paradigm in the search for structural information

Due to its size and stability and its retention of its function when it is solubilized in detergent, EmrE is a unique experimental paradigm for the biochemical and biophysical studies of membrane-based ion-coupled transporters [1–5]. Study of this small, 110-residue multi-drug transporter from *Escherichia coli* has provided valuable information for the understanding of the coupling mechanism of the ion-coupled transporter family [3,6,7]. Biochemical analysis shows that these proteins function by exchanging ions (protons in the case of EmrE) for substrate, which subsequently leads to transport of the substrate across the cell membrane [3]. However, the structural information that became available for this protein in recent years has been in conflict with the existing biochemical knowledge [8,9]. Although the two X-ray crystallography papers for the protein [10] and a prior structure for the protein obtained by electron crystallography of 2D crystals [11] have recently been retracted, their publication sparked a controversy regarding the relative topology of the protomers in the functional dimer, and this controversy is still ongoing [12–14]. The biochemistry of my own group (discussed here) indicates a symmetrical relationship between the monomers in the EmrE homodimer, but genetics and the remaining valid structure for the protein obtained by electron crystallography indicate an asymmetrical relationship and a 'dual topology' relative to the membrane. Can these apparently contradictory results be reconciled, and what impact has the publication of incorrect structures had on researchers in this and other fields?

## Introduction to the biochemistry of EmrE

EmrE extrudes a range of positively charged aromatic drugs in exchange for two protons, thus rendering bacteria resistant to these drugs [1,4,15,16]. Between 1995 and 2000, the protein was characterized, purified and reconstituted in a functional form [1,17,18]. Since then, structural and biochemical evidence has indicated that the basic EmrE oligomer is a dimer [11,19–21].

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 seems to reflect with high fidelity its catalytic activity in the membrane. This has enabled a detailed study of the molecular basis of substrate recognition and the coupling between protons and substrate [3,6,7,22–25]. EmrE has only one membrane-embedded charged residue, Glu14, which is also conserved in >200 homologous proteins in bacteria and archaea [26] (Figure 1). Glu14 provides the core of the coupling mechanism because its deprotonation is essential for substrate binding [3,17]. Conversely, substrate induces proton release and both reactions (substrate binding and proton release) have been observed directly in the detergent-solubilized preparation of EmrE [7] (Figure 1b). The estimated acid dissociation constant ($pK_a$) for Glu14 is unusually elevated (~8.5 compared with 4.25 for the same carboxyl in aqueous environment), indicating that the environment around Glu14 is chemically unique. The fact that the binding site for both substrates and protons overlap and that its occupancy is mutually exclusive provides the basis of the coupling mechanism [3,6]. The fine-tuning of the $pK_a$ is essential because replacement of Glu14 with aspartic acid results in a decrease in the $pK_a$ of the carboxyl to 6.5 and generates a protein that, at physiological pH, has already released the previously bound protons, binds substrate but cannot couple the substrate flux to the proton gradient [7,22].

A possible explanation for the special environment that leads to this finely tuned and high $pK_a$ might stem from experimental data that imply a role for at least three aromatic residues (Trp63, Tyr40 and Tyr60) in each EmrE monomer (Figure 1a). Our results indicate that, in the absence of substrates, the carboxyls of Glu14 in the binding cavity are stabilized by interaction with protons or with at

*Corresponding author:* Schuldiner, S. (Shimon.Schuldiner@huji.ac.il).
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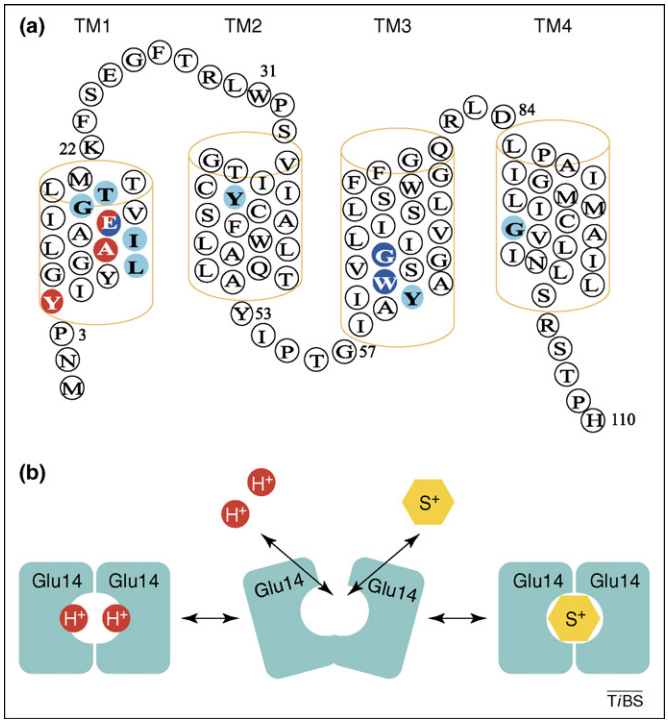


**Figure 1**. Secondary-structure model of EmrE and the mechanism of coupling protons and substrate fluxes. The predicted secondary structure for EmrE and the likely mechanism for the coupling of protons and substrate fluxes are illustrated. **(a)** The essential residues in EmrE are highlighted in blue. Residues that affect coupling are shown in red. Glu14 is shown with both colors because it is an essential residue, but replacement with aspartic acid generates an uncoupled protein. Residues that affect substrate specificity or affinity or that can be at least partially replaced with specific replacements are shown in light blue. For the sake of clarity, this is not a comprehensive review of each mutation that affects activity, and only selected residues exhibiting the strongest effects and that are discussed in the text are marked. **(b)** The molecular basis of coupling substrate and protons fluxes in EmrE was studied using detergent-solubilized protein and following proton release upon substrate binding to the negatively charged species [3,7,22,27]. Binding sites for protons and substrate overlap. Deprotonation of the glutamic acid residues at position 14 is necessary to enable substrate binding. The high $pK_a$ of the carboxyl at position 14 is essential for coupling of fluxes of protons and substrate.

least part of the six aromatic residues (three from each monomer) [27,28]. The aromatic residues provide an environment that might explain the unusually high $pK_a$ values of these carboxyls and enable interaction with the hydrophobic substrates, as has been documented in other proteins that bind similar substrates [29,30].

## What is the topology of EmrE?

Before discussing the topology of EmrE and the experiments that have been performed in an attempt to determine it (Box 1), it is necessary to first define the nomenclature used here. It is evident that there is the potential for a dimeric membrane protein to adopt different topologies. Relative to each other, the two protomers can theoretically adopt either a parallel (e.g. see the homodimers in Figure I, Box 2) or antiparallel orientation (e.g. see the heterodimers in Figure I, Box 2). In addition, relative to the lipid bilayer, the dimers can theoretically adopt a single topology or a dual topology where, for example, parallel dimers are either all in a $C_{in}$ (or $C_{out}$) orientation or a mixture of both $C_{in}$ and $C_{out}$ (note, $C_{in}$ and $C_{out}$ represent the location of the C terminus of the protomers relative to the cell contents). However, are all the potential topologies feasible in a biological sense? The answer to that question must be no because there are constraints such as the way the protein is inserted into the membrane during synthesis and the requirement for the dimer to exist in a catalytically active form. For that reason, determining the topology of EmrE is essential not only for learning how it functions but also for learning how the protein has evolved. As more complex membrane proteins could have evolved from smaller membrane proteins (Box 2), this also has implications for the evolution of membrane proteins in general.

### Box 1. Determining the topology of membrane proteins

The topology of a membrane protein is determined by the interaction between the sequence of the protein itself, the composition of the lipids in the membrane and the insertion machinery [50–52]. The positive inside rule recognized that the cytoplasmic sides of membrane proteins contain an excess of positively charged residues and has proven a useful tool for predicting the topology of bacterial membrane proteins [53]. von Heijne and collaborators have presented the topology-determining influence of positive charge by showing that, by altering the distribution of charged residues, the topology can be changed [54]. A variety of techniques have been developed for experimental testing of the topology of individual proteins. Topology reporters that can fold into active conformation only when present either in the periplasm or the cytoplasm have proven extremely useful tools but there are limitations in specific cases (discussed here) [43,44]. The suggestion of dual topology of some proteins received recent attention from global-topology analyses using such reporters [39,42]. Although these results might reflect the limitations of the technique, they might also indicate that, in some cases, the topology determinants are not absolute and given proteins might be in equilibrium between two topologies. However, in the case of homo-oligomeric proteins such as EmrE, the use of reporters cannot distinguish between dual topology (a mix of both $C_{out}$ and $C_{in}$ dimers) and antiparallel topology that describes the arrangements of the monomers relative to one another within the dimer. For demonstrating antiparallel topologies (if they exist) additional approaches are needed.

*Earlier biochemistry indicates structural symmetry...*

Several experimental approaches support the 'biochemical symmetry' of EmrE, that is, the complete equivalence of the residues in both monomers. Early biochemical data from our laboratory (published in 2004) demonstrate the same topology relative to each other for all protomers in the intact cell and in membrane vesicles [31]. Complete equivalence of residues in both monomers was demonstrated in several studies. Thus, using electrospray ionization mass spectrometry it was directly demonstrated that the two essential Glu14 residues in the dimer are quantitatively (>80%) modified by carbodiimides in a way that indicates that they are both in a similarly hydrophobic environment [25]. Moreover, tetraphenylphosphonium ($TPP^+$), a substrate that binds EmrE with high affinity, reduces modification of Glu14 by ~80%, indicating that both Glu14 residues in the functional unit are close enough to the binding site that $TPP^+$ prevents their modification or that they might be equivalently protected in an allosteric manner by $TPP^+$ [25]. The results suggest that the Glu14 residues in the EmrE dimer are functionally equivalent. In another study, chemical modification of cysteine replacements in the same transmembrane (TM) domain strongly supports the same conclusion: four residues in the TM are fully

**Box 2. Topology evolution might have been directed by a need to extend substrate specificity**

Conceivably, the evolutionary challenge of recognition and transport of a wide spectrum of substrates might have selected for small multidrug transporter (SMR) heterodimers that originated from gene duplication of the more ancient homodimers. The number of new combinations (and therefore new and wider spectrum of substrates) possible with heterodimers is undoubtedly higher than those that can be obtained in a homodimer. After gene duplication, few mutations in the hydrophilic domains might be sufficient to convert a homodimer into a heterodimer and vice versa. In this manner, one protein with only a slightly modified sequence could extend the range of the substrate specificity. Topology evolution of larger proteins can now be visualized starting from the SMR heterodimers, which can then fuse and give rise to larger proteins, some of them such as LacY, GlpT and AcrB [55–58] with domains in parallel orientation, others such as aquaporins, ClC channel and the neurotransmitter transporter homolog, LeuT [36–38], with two oppositely oriented membrane domains. Most of the potential heterodimers identified *in silico* have a strong and opposite charge bias, whereas others do not (Figure I).

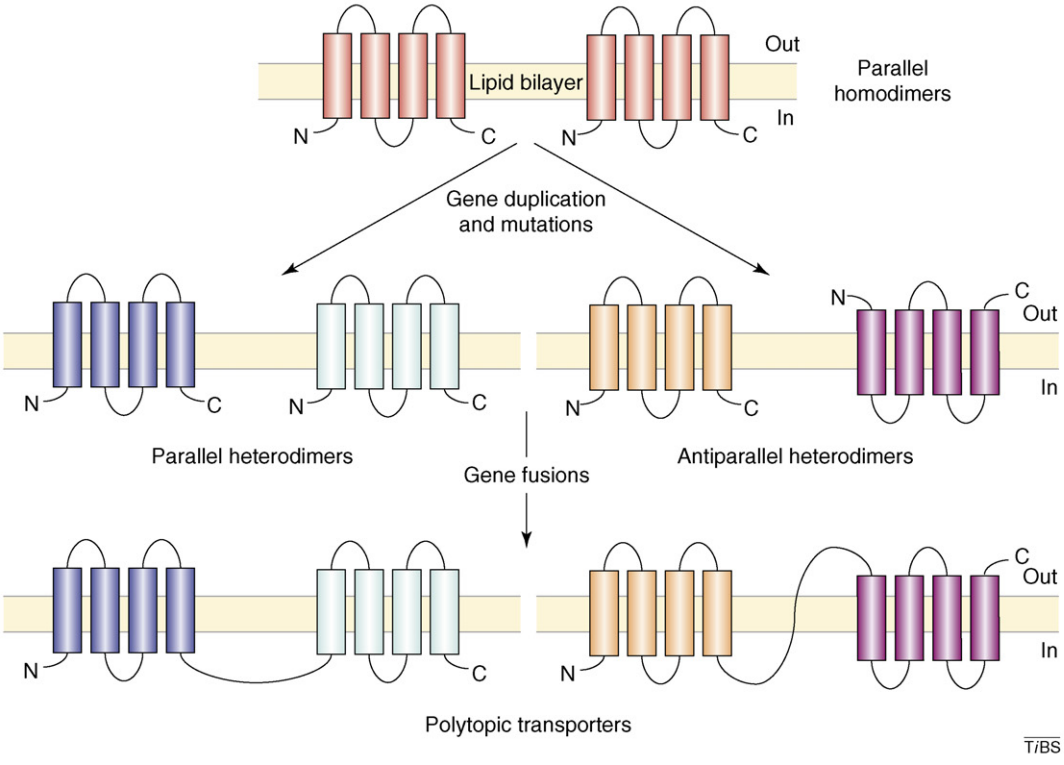


**Figure I.** Evolution of membrane proteins. The potential process for the evolution of more-complex polytopic membrane proteins from a small, parallel homodimeric membrane protein.

accessible to alkylation with maleimides and two of them are also fully protected by substrates [26]. The accessibility pattern indicates a scissor-like packing of the two TM1s (transmembrane segment 1) in a parallel topology (i.e. the arrangement shown in Box 2 for the homodimer) [26]. These findings are in good agreement with a model proposed in 2003 by Koteiche *et al.* [32] using site-directed spin labeling.

*...but structural data might imply asymmetry...*

In 2003, the 3D structure of EmrE with and without bound $TPP^+$ was determined at 7.5-Å resolution by cryo-electron microscopy (cryo-EM) of 2D crystals [11,33]. As will become evident, it is notable that the two structures are practically indistinguishable. The minimal structural unit is an asymmetric homodimer composed of eight transmembrane α helices in total (i.e. four helices from each monomer), with density for $TPP^+$ in a binding chamber formed by six out of the eight α helices, confirming the suggestion that $TPP^+$ binds near the center of the dimer [11]. Subsequent to the publication of these electron crystallographic structures, two higher resolution X-ray crystallography structures of EmrE with and without the high-affinity substrate $TPP^+$ were published in 2004 and 2005, respectively. Suprisingly, the two structures were very different from each other [8,9]: the first was a dimer of dimers, in which each protomer had an antiparallel topology and one of the TM domains in each protomer was not properly folded; the second was a dimer with each protomer in an antiparallel topology relative to each other. The presence of substrate in the second structure could have been responsible for the large differences between the two structures, although similar substrate-induced conformational changes had not been observed in the structures obtained from the 2D crystals. The X-ray structures also exhibited an asymmetric dimer, with the protomers possessing an antiparallel topological orientation. Both X-ray structures have since been extensively criticized in several experimental and theoretical papers by us and other groups in the field [7,12,14,16,25–28,31,34,35].

Recently, the X-ray structures were retracted because it was found that faulty software had been used to solve them [10]. However, before these retractions, the suggestion of an antiparallel arrangement of the monomers in a

homodimeric membrane protein attracted widespread interest because of the intriguing implications regarding biogenesis, insertion and evolution of ion-coupled transporters, and it deserved careful attention. The finding also had obvious and exciting similarities to the internal structural repeat found in several membrane proteins such as aquaporins, ClC channel and the neurotransmitter transporter homolog, LeuT [36–38].

The claim for an antiparallel topology has been supported by a reinterpretation of the afore-mentioned electron-density maps of 2D crystals of EmrE that showed that parts of the structure are related by quasi-symmetry [11]. The authors used this symmetry relationship, combined with sequence conservation data, to assign the transmembrane segments in EmrE to the densities seen in the cryo-EM structure. A Cα model of the transmembrane region was constructed by considering the evolutionary conservation pattern of each helix. The model is validated by much of the biochemical data on EmrE, with most of the positions that were identified as affecting substrate translocation being located around the substrate-binding cavity [12].

### *...however, a parallel topology is fully functional!*

The antiparallel arrangement of the protomers in the EmrE dimer first suggested by the structural data (and subsequently backed by the genetic approach [13,39]; discussed later), is at odds with the biochemical data supporting the complete equivalence of residues in both monomers with a scissor-like packing of the two TM1s. In addition, previous crosslinking studies are consistent with a parallel topology of the two monomers in the EmrE dimer [40]. Experimental constraints provided by crosslinking experiments might be limited in that they provide a snapshot of only one possible conformation, which might not be physiologically relevant. In addition, chemical modification of the protein required for crosslinking might cause misfolding or denaturation. Therefore, results obtained from crosslinking experiments must be evaluated with caution. For these reasons, we used a most stringent criterion: functionality after crosslinking. We introduced cysteine replacements in TM4 at positions too far apart to crosslink in an antiparallel structure (~35–45 Å). Despite this distance, and as expected from a parallel topology, efficient crosslinking was observed with o-phenylenedimaleimide (o-PDM) [14], a rigid, bifunctional crosslinking agent that is 7–11 Å long [41]. The choice of replacements was based on previous experiments indicating that the dimer interface within the EmrE is formed by contacts between TM1 and TM4 [40]. In addition, D84C and T108C, the two single-cysteine replacements used, at both the N and C termini of TM4, are fully functional with respect to phenotype, ligand binding and transport, and are, therefore, likely to represent the functionally tertiary structure [23].

Crosslinking of monomers mediated by o-PDM at positions 84 and 108 is not consistent with an antiparallel topology [14]. A large fraction of EmrE, particularly the single-Cys108 mutant, was crosslinked (60–70%), and the crosslinked protein was further purified (~95%) by ion-exchange chromatography. The purified protein crosslinked at position 108 displays substrate binding with properties almost identical to that of the non-crosslinked species. The affinity for $TPP^+$ and the number of binding sites detected (0.84 molecules of ligand per EmrE dimer) indicate that almost all the protein is functional. After reconstitution into proteoliposomes, the T108C crosslinked protein displayed $H^+$-driven transport of methyl viologen at rates and to levels nearly identical to those displayed by the non-crosslinked species [14].

The crosslinked protein is fully functional and almost every dimer binds ligand, as shown by the finding that it binds approximately one molecule of $TPP^+$ per EmrE dimer. However, to test the possibility that the functional unit might be formed by the interaction of two crosslinked dimers, we used two different approaches [14]:

Because it is most likely that the first stage of the thermal denaturation of EmrE is dissociation of the dimer, it seems reasonable to suggest that stabilization of the dimer by crosslinking will result in stabilization of EmrE functionality. Indeed, crosslinked T108C is remarkably more stable with respect to heat denaturation than the untreated protein, and retains almost 90% activity even after 6 h at 80 °C.

Monomer swapping is induced by agents such as temperature and some detergents that reversibly affect the oligomeric state of EmrE [14,21] (Box 3). When functional proteins such as wild-type or the untreated T108C and D84C mutant proteins are mixed with an excess of the inactive mutant EmrE $E^{14}C$, the mixed dimers display a 20-fold lower affinity for substrate [21]. At both the 84 and

**Box 3. Membrane proteins: a soap opera?**

In some cases, the question is raised whether a given reported conformation is an experimental artifact created by the different milieu the proteins face when removed from their native environments. This might turn out to be especially crucial for membrane proteins, for which we can only moderately mimic the original conditions after solubilization with detergents. This, for example, has been the rationale in the case of the voltage sensor of the $K^+$ channel $K_v$AP, where it seems that the detergent micelle is a poor mimic of the membrane and that lipids are essential for maintaining the structure of the voltage-sensor domain [59]. Other cases, for example, the *Escherichia coli* $Na^+/H^+$ antiporter NhaA [60], the archaeal protein-conducting channel [61], the mitochondrial adenine nucleotide translocator [62], crystallized as monomers despite biochemical and biophysical evidence of oligomeric structures in the membrane [63–66].

A crucial consideration is whether or not the structure in detergent is in a physiological state, and this is difficult to assess directly. EmrE is unique because it provided assays for substrate binding and proton release in the detergent solubilized form. When using the proper detergent, the protein binds $TPP^+$, and other substrates, with high affinity [17]; substrate induces stoichiometric release of hydrogen ions [7] and many of the findings described with the detergent-solubilized protein have been confirmed with the protein reconstituted in proteoliposomes [16,23] Obviously, detergents might more easily affect the oligomeric state before affecting the tertiary structure. In the case of EmrE, this issue has been studied in detail: the dimeric structure is sensitive to the nature of the detergent used [14]. Whereas n-dodecyl-β-maltoside-solubilized EmrE dissociates only under extreme conditions, solubilization in octyl-β-glucoside or nonyl-β-glucoside – the detergents used for crystallization – increases the fraction in the monomeric form [14]. Monomers might form crystal contacts that are different from those in the functional protein. For example, although LacY is a monomer both structurally and functionally (for review, see Ref. [67]), the asymmetric unit in the crystal lattice contains a dimer with the monomers in opposite orientation [55].

108 positions, o-PDM crosslinking prevents EmrE from entering into oligomerization equilibrium and exhibits a degree of activity directly correlated with the degree of crosslinking [14].

Crosslinking between dimers rather than within the dimer was also ruled out. If crosslinking occurred more than once it would generate tetramers or even higher-order oligomers. However, this is not the case. The crosslinked dimer is >90% pure and, furthermore, crosslinked, and the untreated EmrE behave identically on size exclusion chromatography, showing that large complexes are not generated by crosslinking between dimers [14].

## Does EmrE exhibit single or dual topology?

In addition to the biochemical and structural data, genetic work has been performed that also tackles the issue of the topology of membrane proteins. In 2005, Von Heijne and collaborators performed a comprehensive global analysis of the topology of a large fraction of the *E. coli* membrane proteins [42]. In this study, two topology reporters were used: alkaline phosphatase (PhoA) and green fluorescent protein (GFP). PhoA can fold into an enzymatically active conformation only when present in the periplasm [43]. By contrast, GFP becomes fluorescent only when present in the cytoplasm [44]. Thus, the location of the C terminus of a membrane protein can be determined by making PhoA and GFP fusions at the C terminus: high PhoA with low GFP activity indicates periplasmic location, whereas a low PhoA and high GFP activity indicates cytoplasmic location. C-terminal location ($C_{in}$ and $C_{out}$) was determined in this manner for an impressive 502 of the 665 cloned proteins. For 69 out of 71 proteins, the C-terminal assignment agreed with previously published conclusions. Using these reporters, EmrE was unambiguously assigned as $C_{out}$. The rationale behind the suggestion that EmrE might have a dual topology was that its GFP fluorescence is higher than background, but this was actually the case for at least 25–30 other proteins (as observed Ref. [42]). The authors suggest that this is the case when the results are taken at face value, but the C-terminal GFP moiety biases the results by causing a shift towards the $C_{out}$ orientation [13]. To support the concept of dual topology, mutational studies were performed that showed that the topology of the EmrE fusion proteins in the membrane is sensitive to the distribution of positive charges in the protein [39]. In addition, manipulation of the positive charges generates a set of mutants, some with $C_{out}$, others with $C_{in}$ topology [13,39]. Two of these mutants, bearing three mutations each, have been shown to be inactive. Co-expression of the inactive mutants restores the ethidium-bromide-resistance phenotype to the same level as seen with wild-type EmrE [13]. The authors conclude that these results strongly indicate the formation of a functional, antiparallel heterodimer. Although this is certainly a possible interpretation, more direct information, beyond phenotypic complementation, is needed to support this contention. In addition, six mutations (three in each monomer) might have pushed EmrE to form a hetero-oligomer and, if this is indeed the case, it is an elegant demonstration of the evolutionary case made by the authors (presented in a markedly modified form in Box 2). However, it remains to be directly demonstrated whether the protein that has not been mutated can form functional homodimers with an antiparallel topology. Moreover, the existence of homodimers with antiparallel orientation would pose a problem for the insertion machinery of membrane proteins. Identical protomers with $C_{out}$ and $C_{in}$ topologies would insert at exactly a 1:1 ratio to prevent the existence of unpaired units. How this ratio is controlled and how the assembly of such a dimer is achieved is difficult to envisage with the present knowledge.

The topological studies of tagged overexpressed EmrE in intact cells in our laboratory indicate that all the protein is in a $C_{in}$ configuration [31]. The studies using PhoA and GFP fusions suggest that all the fused 'wild-type' protein is in a $C_{out}$ configuration [39]. The discrepancy in the topology of the 'wild type' between the two groups might stem from the diversity of the constructs, the fusion tags and/or the strains used. Although our laboratory has added a Myc epitope before a six-histidine tag at the C terminus [17], others have added a six-histidine tag and protease cleavage sites at the N terminus [8,9], and others have added large reporters such as PhoA and GFP at the C terminus [39]. The difference in the results with the various constructs might be a warning about the bias that, in certain cases, tags and reporters might introduce to topology determinations. Elucidation of the arrangement of the dimer relative to the membrane requires more detailed study and, presently, the answer to the question of the topology of EmrE remains unresolved.

## Antiparallel topology in heterodimeric SMRs?

A case for antiparallel topology has been made for another group of proteins in the small multi-drug transporter (SMR) family [39]. It has been suggested that members of this group of SMR proteins function as hetero-oligomers based on the finding that some pairs confer a resistance phenotype only when co-expressed [45,46]. A bioinformatic analysis of this group of proteins indicates that the distribution of positive charges is different in a way that would predict a topology of opposite direction for each protomer (i.e. antiparallel) [39]. We suggest that the evolutionary challenge of recognition and transport of a wide spectrum of substrates might have selected for SMR heterodimers that originated from gene duplication of the more ancient homodimers. After gene duplication, few mutations would enable them to assume either parallel or antiparallel orientation of the monomers within the heterodimer [13,47]. In this manner, one protein with only a slightly modified sequence might extend the range of the substrate specificity. Topology evolution of larger proteins with two oppositely oriented membrane domains can now be visualized starting from gene duplication, mutations and then fusion of SMR heterodimers (Box 2). If it turns out that a few mutations in the hydrophilic loops transform a functional parallel homodimer to a functional antiparallel heterodimer [13] and vice versa [47], it raises a fascinating question of what the minimal requirement is for catalysis of ion-coupled transport. The binding cavity of the parallel and antiparallel dimer is now very different but it still has one basic component: two charges in a highly hydrophobic environment formed by, in the case of EmrE, six aromatic

residues. Is this enough to ensure the vectorial movements of protons and substrates? This is an intriguing question that awaits more detailed studies.

If the case can be made for antiparallel heterodimers, what makes it so different for a homodimer? As mentioned, if antiparallel homodimers were to exist, this would pose intriguing questions about the insertion and assembly of these proteins in the membrane. In addition, our current findings are consistent with the fact that EmrE with a parallel arrangement of the protomers in the dimer is fully functional as judged by its ability to bind substrate and transport it against an electrochemical gradient in exchange with protons [14]. The case for antiparallel topology is supported by the studies from von Heijne's laboratory [13,39] and by the low-resolution Cryo-EM structure that was recently used to derive a Cα-model structure [12]. However, the functionality of EmrE and close homologs with an antiparallel orientation of the monomers has not yet been biochemically demonstrated.

## Macromolecular models and experimental findings

The case for antiparallel homodimers was initiated by the two X-ray structures of EmrE. Just before submission of this review, the retraction of five structures of membrane proteins published by the Chang laboratory was announced: three structures of MsbA, an ABC transporter and both structures of EmrE (discussed here) [10]. Surprisingly, few reactions dealt with this quite dramatic and unusual episode and most of them dealt only with the software problems that led to this retraction (see Ref. [48] and http://www.ccp4.ac.uk/ccp4bb.php, but see also Ref. [49]). Because of the wrongly assumed omnipotence of the crystallography data, many of the biochemists working in the field were faced with a need to perform additional time-consuming experiments to make a case for statements and conclusions in publications and grants. In addition, high-visibility journals were not keen to publish views that were in disagreement to those already strongly supported by structural data. Scientists must ask whether and how we can try to prevent this from happening again. Macromolecular models are supplying extraordinary tools for a mechanistic understanding at a molecular level of many biological processes. However, it must not be forgotten that confidence in the validity of a structural model is justified to the extent that the model fits known observations and makes predictions that are verifiable. The situation with EmrE and MsbA presented an instance in which the biochemical findings (and, to an extent, the prior lower-resolution structure obtained from electron crystallography) provided a strong reason for questioning the veracity of the structural model. Even if a structure is crystallographically 'correct', it is not necessarily physiologically relevant. With regard to membrane proteins, it seems trivial to emphasize that detergents only mimic the milieu of the membrane and that this is sometimes overlooked (Box 3). It is no wonder that many of the first high-resolution structures were derived from respiratory and photosynthetic complexes that must be highly stable to enable efficient electron transfer or from other extremely stable proteins such as β barrels or bacteriorhodopsins.

## Concluding remarks

The story of the structure and topology of EmrE is still ongoing. A small, stable and unique protein, fully functional in the detergent solubilized state, has unveiled basic questions of coupling between substrate and ion fluxes, has yet to expose its structure at high resolution. Is it a parallel, single-topology homodimer or an anti-parallel, dual-topology homodimer? The structure was retracted owing to faulty software, and the structures might be republished once the software issue has been resolved. Indeed, this has been done for three structures of MsbA and the revised PDBs have been deposited two months before retraction. It will be interesting to see what the relationship will be like between the monomers once the EmrE structure is recalculated. The controversies over its structure and topology might reflect the fact that the functional unit is a dimer and that oligomeric interactions are highly sensitive to the detergent used in its study. In addition, we might be at an 'evolutionary junction', where the need to expand the range of substrates of this multidrug transporter could only be met with the higher number of combinations that are possible in heterodimeric proteins. The problems faced by insertion of small proteins where the dimer is the functional unit might also account for why, in the evolutionary process, these proteins vanished in the competition with their larger analogs and were left only for scientists that are actively in search of easy to use experimental paradigms.

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