

EmrE, THE SMALLEST ION-COUPLED TRANSPORTER, PROVIDES A UNIQUE PARADIGM FOR STRUCTURE–FUNCTION STUDIES

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Summary

EmrE is an *Escherichia coli* multidrug transporter which confers resistance to a wide variety of toxicants by actively removing them in exchange for hydrogen ions. EmrE is a highly hydrophobic 12 kDa protein which has been purified by taking advantage of its unique solubility in organic solvents. After solubilization and purification, the protein retains its ability to transport as judged from the fact that it can be reconstituted in a functional form. Hydrophobicity analysis of the sequence yielded four putative transmembrane domains of similar sizes. Results from transmission Fourier transform infrared measurements agree remarkably well with this hypothesis and yielded α -helical estimates of 78 % and 80 % for EmrE in CHCl₃:MeOH and 1,2-dimyristoyl phosphocholine, respectively. Furthermore, the fact that most of the amide groups in the protein do not undergo amide–proton H/D exchange implies that most (approximately 80 %) of the

residues are embedded in the bilayer. These observations are only consistent with four transmembrane helices. A domain lined by Cys41 and Cys95 accessible only to substrates such as the organic mercurial 4-(chloromercuri)benzoic acid has been identified. Both residues are asymmetric in their location with respect to the plane of the membrane, Cys95 being closer than Cys41 to the outside face of the membrane. In co-reconstitution experiments of wild-type protein with three different inactive mutants, negative dominance has been observed. This phenomenon suggests that EmrE is functional as a homo-oligomer.

Key words: multidrug transporters, structure, cysteine, MiniTexans, drug resistance, energy coupling, H⁺ transport, methyl viologen, *Escherichia coli*.

Introduction

Resistance to a wide range of cytotoxic compounds is a common phenomenon observed in many organisms throughout the evolutionary scale. In many cases, this resistance is achieved through the action of multidrug transporters, which actively remove a wide variety of toxicants in an energy-dependent process and decrease the concentration of the offending compounds near their target. The proteins responsible for performing this task have been found in organisms from bacteria to man. From analysis of their structure and properties, several families can be distinguished (for a review, see Schuldiner *et al.* 1995). One of them (Smr or MiniTexans) is represented by very small proteins, approximately 100 amino acids long, which render bacteria resistant to a variety of toxic cations (Grinius *et al.* 1992; Paulsen *et al.* 1996). Being the smallest putative biological transporters known, they arouse our attention since they could provide a simple model to study. A variety of genes coding for MiniTexans have been identified in several bacteria on the basis of their ability to confer resistance to several drugs (Grinius *et al.* 1992; Paulsen *et al.* 1996). One of these genes, *emrE* or *mvrC*, is an *E. coli* gene that has been identified and cloned on the basis of its ability to confer resistance to ethidium

(Purewal, 1991) and to methyl viologen (Paraquat) (Morimyo *et al.* 1992). Previous studies have suggested the existence of an efflux system for toxic cationic compounds in *E. coli* (Midgley, 1987) and, because of the hydrophobic nature of the predicted polypeptide, it was proposed that EmrE is indeed an efflux system (Purewal, 1991). The predicted sequence of EmrE suggests that it is a highly hydrophobic 12 kDa protein. A hydropathic analysis of the sequence reveals the presence of four putative transmembrane segments with only one charged residue (Glu) in the putative transmembrane domain and a total of eight charged amino acids throughout the protein (Fig. 1).

Purification and reconstitution of Smr and EmrE

EmrE (Yerushalmi *et al.* 1995) and its *Staphylococcus aureus* homolog Smr (Grinius and Goldberg, 1994; Paulsen *et al.* 1995) have been characterized, purified and reconstituted in a functional form. Both proteins catalyze H⁺-driven cation antiport in proteoliposomes reconstituted with purified transporter and behave as multidrug transporters capable of recognizing a wide range of substrates and inhibitors. Flagged-Smr has been purified after detergent extraction and

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chromatography in an affinity column (Grinius and Goldberg, 1994). Reconstitution was achieved by detergent dilution, and the proteoliposomes were assayed by either methyltriphenyl phosphonium or ethidium bromide uptake.

EmrE has been purified taking advantage of its unique properties of solubility in organic solvents such as a mixture of chloroform and methanol (Yerushalmi *et al.* 1995). After solubilization in this solvent, the protein retains its ability to transport as judged from the fact that it can be reconstituted in a functional form. After overexpression of EmrE and extraction of cell membranes, the organic solvent mixture contains a highly enriched transporter fraction with very few other impurities. The protein has been further purified to homogeneity with a single chromatography step (M. Lebediker and S. Schuldiner, in preparation). The purity has been demonstrated by direct sequencing and by nuclear magnetic resonance analysis of the protein (M. Schwaigler, M. Lebediker, H. Kessler and S. Schuldiner, unpublished results). This organic solvent fraction can be dried in the presence of *E. coli* phospholipids and kept for long periods. The dry protein can be reconstituted by addition of aqueous solvent containing ammonium chloride (Fig. 2). After a freeze and thaw cycle, the proteoliposomes are sonicated in a bath-type sonicator and diluted into an ammonium-free medium. Under these conditions, the highly permeant ammonia moves down its chemical gradient and changes the equilibrium in the vesicle interior so that ammonium dissociates and generates protons, creating a pH gradient. This pH gradient is utilized by EmrE to accumulate substrates such as ethidium bromide and methyl viologen. Uptake of the former can be followed continuously in proteoliposomes preloaded with DNA (Yerushalmi *et al.* 1995). Thus, upon entry of ethidium to the vesicle, its fluorescence quantum yield increases on binding to the nucleic acid.

Secondary structure

Hydrophobicity analysis of the sequence of EmrE yielded four putative transmembrane (TM) domains of similar sizes: TM1, Y4–M21; TM2, L30–I54; TM3, I58–G80; and TM4, L85–S105. The transmembrane domains account for 86 amino acids or 78% of the total residues of the protein. Extramembranous residues are predicted to exist in short connecting loops and therefore not to be in a helical configuration. Thus, one can equate the percentage of membrane-embedded residues to helical content. Results from transmission Fourier transform infrared (FTIR) measurements agreed remarkably well with this notion and yielded α -helical estimates of 78% and 80% for EmrE in CHCl_3 :MeOH and 1,2-dimyristoyl phosphocholine (DMPC), respectively (Arkin *et al.* 1996). The fact that the protein retains its secondary structure in a solution of CHCl_3 :MeOH agrees with the studies performed with subunit *c* of the H^+ F_1F_0 ATPase (Girvin and Fillingame, 1993, 1995) and bacteriorhodopsin (Orekhov *et al.* 1994), which have been documented to retain their secondary structure in this solvent mixture.

The very high helical content of EmrE precludes any other sort of secondary structures, and the length of the sequence of EmrE (110 amino acids) discounts any number of transmembrane α -helices greater than four. Furthermore, the fact that most of the amide groups in the protein do not undergo amide–proton H/D exchange implies that most (approximately 80%) of the residues are embedded in the bilayer. These observations are only consistent with four transmembrane helices.

The fact that the α -helices are transmembrane and not peripheral to the bilayer can be directly verified using attenuated total reflection FTIR (ATR-FTIR). The derived tilt angle (i.e. angle with respect to the bilayer normal) for EmrE in DMPC vesicles from the bilayer normal is 37° . The calculation of the tilt angle does not take into account any bilayer disorder and must therefore be considered as the maximal tilt angle. Bilayer disorder can be estimated by comparing the inherent order parameter of a model membrane ($S=0.95$) with the order parameter measured in this study for the lipid acyl chains ($S=0.7$). The ratio between the two ($S=0.73$) represents the bilayer disorder. Thus, dividing the calculated order parameter for EmrE in DMPC ($S_{\text{amide,I}}=0.5$) by the bilayer order parameter yields the corrected protein order parameter of $S=0.68$ and subsequent tilt angle β of 27° . As FTIR measures properties of the system as a whole, the tilt angle calculated above should be considered as the average tilt per helix.

So far the results indicate that EmrE is a bundle of four transmembrane α -helices each roughly 20–25 amino acids in length, with an average tilt β of 27° . The tilt angle of the helices in EmrE is similar to that measured for bacteriorhodopsin (26°), agreeing with the structure solved by cryo-electron crystallography. In cytochrome *c* oxidase, most of the transmembrane helices are not vertical to the membrane plane and they are usually not parallel to each other. The crossings between nearest-neighbor helices occur in any of the three types of helix–helix interactions. It is instructive to compare the results of our study with high-resolution structures of soluble proteins possessing this motif.

The most studied proteins sharing the motif of an anti-parallel four-helical bundle are Rop (Banner *et al.* 1987), cytochrome *b562* (Lederer *et al.* 1981), cytochrome *c'* (Finzel *et al.* 1985) and myohemerythrin (Sheriff *et al.* 1987). All of these proteins are of similar size (Rop being the exception since it is a dimer, each protomer contributing two helices) with similar average helix lengths. The length of the helices in EmrE, a parameter influenced by the dimensions of the bilayer, is similar to that found in the soluble proteins.

What distinguishes EmrE from the soluble proteins is the relative tilt of the helices from the long axis of the complex. In the soluble proteins, this angle is less than 15° whereas in EmrE the measured tilt angle is 27° . The tilt angle between the helices is slightly increased in the cofactor-binding proteins (heme in the case of both cytochromes or iron in the case of myohemerythrin) as opposed to that found in Rop. This phenomenon may be due to the cavity formed upon splaying

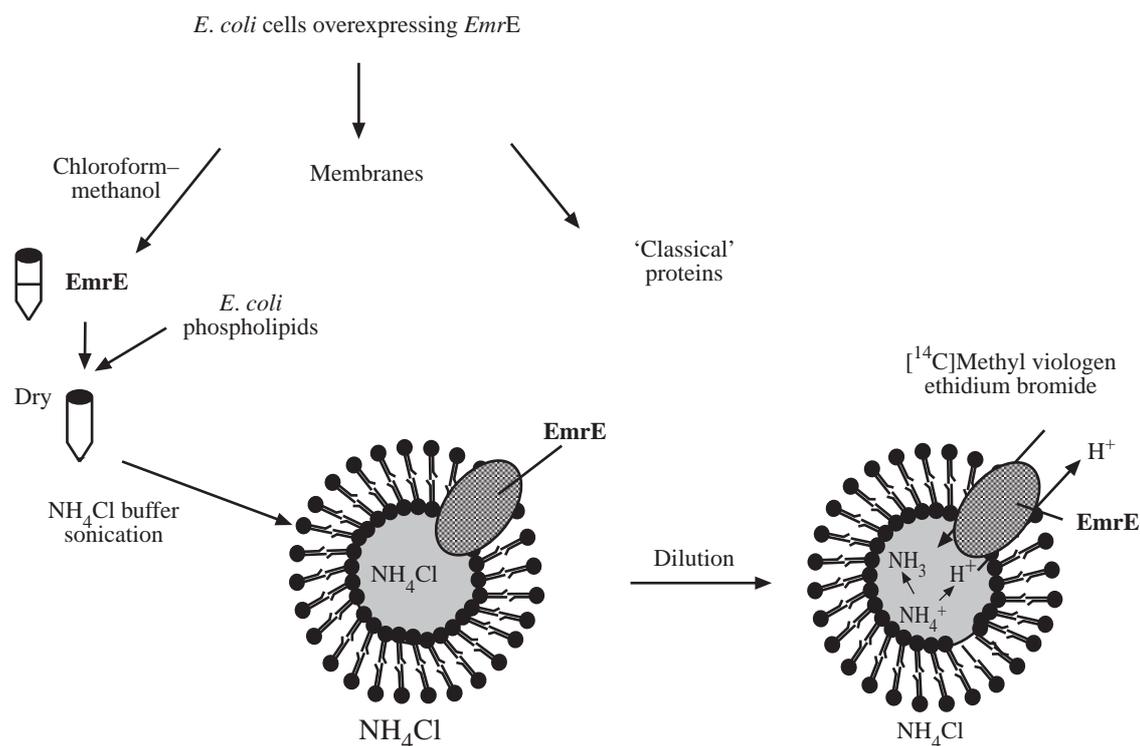


Fig. 2. Scheme for the purification and reconstitution of EmrE. Solubility in organic solvents is a unique property of EmrE and very few other membrane proteins. Therefore, overexpression of EmrE and extraction of membranes with organic solvents yields a highly enriched preparation which can be easily reconstituted (see text for details).

is mixed (Fig. 3B) and practically random, approximately 30–50% in the configuration similar to that observed in whole cells (right side out, C95 exposed), and the other 50–70% in an inverse configuration (inside out, C41 exposed). Taken together, the results suggest that Cys41 can be reached by PCMBs only from the membrane face equivalent to the internal side of the cell (Fig. 3A). Cys95 is fully accessible in intact cells but, since the accessibility in proteoliposomes is only partial, we conclude that it is accessible exclusively from the outside face of the cell.

The reactivity of Cys to small sulfhydryl-reactive agents has been used previously to map the residues lining the channel pathway in several cases (Akabas *et al.* 1994a,b; Xu and Akabas, 1993). Cys residues were systematically engineered in domains previously suspected to line the channel, and their ability to change the conductance properties was recorded. In these cases, the selectivity is determined by the small size and by the charge of the probing reagent, but most of the channel is freely accessible. In other transporters, proximity to the substrate path is inferred from protection by substrates against inactivation. In ion-coupled transporters, an extensively studied case is that of a Cys residue (Cys148) in the lac permease, a β -galactoside/ H^+ symporter from *E. coli*. Substrate protection experiments (Fox and Kennedy, 1965) and biophysical studies (Wu and Kaback, 1994) suggest that Cys148 is located in the vicinity of the substrate binding site.

In ion-coupled transporters, at least part of the permeability pathway must be reachable only by substrates or inhibitors. Yan

and Maloney (1993, 1995) have probed a bacterial membrane antiporter which exchanges glucose 6-phosphate and inorganic phosphate with PCMBs, a hydrophilic and impermeant sulfhydryl-reactive agent presumably resembling the substrate in volume and charge. They showed that transmembrane segment 7 is an α -helix whose central portion is accessible to PCMBs. In the case of EmrE, a unique property of the domain identified is its inaccessibility to compounds other than the substrate, in our case PCMB (Fig. 3A). This domain is lined by Cys41 and Cys95. In addition, we conclude that these residues are asymmetric in their location with respect to the plane of the membrane: Cys95 is closer to the outside face of the membrane and Cys41 is closer to the inner face. On the basis of the findings presented, we suggest the existence in the translocation pathway in EmrE of a domain accessible only to substrates, a 'selectivity filter' to which access is limited. It will be interesting to determine the size of this domain relative to subdomains in which access is unlimited. In this respect, in wild-type Smr, a protein homologous to EmrE from *S. aureus*, there is a single Cys in a position equivalent to position 43 in EmrE. This Cys is accessible to *N*-ethylmaleimide and the inactivation process is slowed down by substrates (Paulsen *et al.* 1995).

Negative dominance studies demonstrate the oligomeric structure of EmrE

Because of its small size, it was particularly interesting to determine the functional oligomeric unit of EmrE. For this

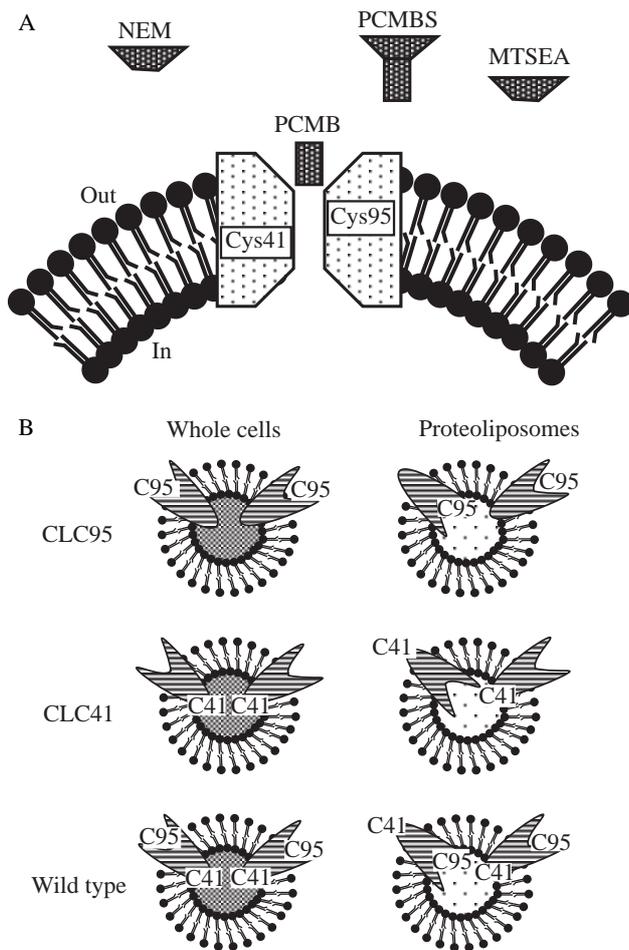


Fig. 3. The substrate translocation pathway in EmrE. (A) Two domains can be distinguished in the substrate translocation pathway: one of them is only accessible to substrates. The relative size of each of the domains has not been determined yet. PCMB, 4-(chloromercuri)benzoic acid; PCMBs, 4-(chloromercuri)benzenesulfonic acid; NEM, *N*-ethylmaleimide; MTSEA, methanethiosulfonate ethylammonium. (B) Studies on the accessibility to impermeant inhibitors such as PCMBs show that residue 95 is accessible to the latter only from the outside, while residue 41 is accessible only from the inside. In proteoliposomes, a random insertion of the transporter yields a mixed topology. CLC95 and CLC41 contain single Cys residues in positions 95 and 41, respectively.

purpose, the effect of several inactive mutants on the activity of the wild-type protein was probed in a mixing approach both *in vitro* and *in vivo* (Yerushalmi *et al.* 1996). In these experiments, various mutants (E14C, Y60F and W63F) were co-expressed with the wild-type protein and shown to decrease significantly the ability of the latter to confer resistance to various toxicants. All the mutants were inactive as judged from their failure to confer resistance to methyl viologen and other toxicants. They also displayed no activity when purified by extraction with organic solvents and reconstituted in proteoliposomes. To document and quantify the *in vivo* effect, the purified mutant proteins were mixed with the wild-type protein. When co-reconstituted with wild-type protein, they

inhibit the activity of the latter in a dose-dependent manner up to full inhibition. We assume that this inhibition is due to the formation of mixed oligomers in which the presence of one nonfunctional subunit causes full inactivation. A binomial analysis of the results based on the latter assumption suggests that the oligomer is composed of more than two subunits, most likely three.

Transport mechanism

No detailed molecular mechanism of transport can be put forward without high-resolution structural information on the proteins catalysing the transport reaction. Indeed, detailed information on mechanisms of proton translocation across membranes is available only for those cases in which structural information is available (Iwata *et al.* 1995; Okamura and Feher, 1992; Lanyi, 1995). In bacteriorhodopsin, it has been suggested that proton transport is accomplished according to an alternating access model of ion pumps in which there is a transition between two protein conformations that allow alternative access of the ion binding site to the two membrane surfaces. The 'switch' allowing this reaction is the break in connectivity of the Schiff base with the cytoplasmic side (Asp96) upon photoisomerization and the unidirectional transfer of a proton to a residue connected to the extracellular face (Asp85) following the decrease in proton affinity of the Schiff base.

In the case of ion-coupled transporters, the mechanism would have to ensure the linkage of the movements of the various substrates in the appropriate direction; for EmrE, the toxic aromatic cation which exchanges with two protons. Evidence is mounting regarding the role in the catalytic cycle of the only charged residue in the transmembrane domain, Glu14 in EmrE. Replacement of the equivalent residue in Smr (Glu13) with Asp and Gln impairs the ability of the protein to confer resistance against ethidium (Grinius and Goldberg, 1994). In EmrE, the mutant protein (E14C) has been purified, reconstituted and shown to be incapable of driving Δ pH-driven transport as well as downhill efflux and exchange (H. Yerushalmi and S. Schuldiner, unpublished results). In addition, wild-type EmrE is inhibited by lipid-soluble carboxy-modifying reagents such as *N,N'*-dicyclohexylcarbodiimide (H. Yerushalmi and S. Schuldiner, unpublished results). The pK of E14 is unknown, and we cannot make educated guesses as all the possible influences of the protein environment on the pK of this residue are unknown. We can, however, expect that in a homotrimer, the mere effect of charge-clustering on the three otherwise identical residues will be such that their pK values may differ very significantly. This may introduce some asymmetry into the oligomer as two of the residues may be permanently protonated whereas the third one maybe charged or neutralised by another ion or residue. We can speculate that, if the positively charged substrate draws near the above cluster, it will modify the pK of the protonated residues, will induce proton release and will form an ion pair. This ion pair will be released only upon arrival of two protons from the opposite side of the membrane. Many questions remain open about the

proposed catalytic cycle: for example, what determines the directionality of proton movement, those released in the first part and those taken up in the second one, and what determines the directionality of substrate release?

The question of the high polyspecificity of the transporter is also still open. What is the basis of an apparent high-affinity recognition of such a wide range of substrates? It is possible that the central anchoring point in the binding site recognizes only the very basic common characteristics of all EmrE substrates: an aromatic cation in which the charge is usually on the ring or very close to it.

Answers to these and many other questions may become apparent when a high-resolution structure of EmrE becomes available.

Physiological role of multidrug transporters

Multidrug resistance is a major concern in medical and agricultural diseases. In medicine, the emergence of resistance to multiple drugs is a significant obstacle in the treatment of several tumors as well as many infectious diseases. In agriculture, the control of resistance to plant pathogens is of major economic importance. However, these traits existed long before the drugs and antibiotics were discovered. Resistance to a wide range of cytotoxic compounds is a common phenomenon observed in many organisms throughout the evolutionary scale and probably developed in order to cope with the variety of toxic compounds that are part of the natural environment in which living cells dwell. Only those organisms that have developed through evolution the ability to cope with a wide variety of compounds have been able to survive. One of the strategies which evolved is removal of toxic substances by multidrug transporters such as EmrE and many others. A question commonly asked about multidrug transporters is what is their 'real' function. Are these proteins functioning solely to protect the organism against toxic compounds or do they have a very specific function and, by chance, happen also to be polyspecific. As in many other cases, the answer seems to be a complex one: clearly, in proteins such as Mdr1a, functioning in the blood-brain barrier (Schinkel *et al.* 1995), or the organic cation antiporter in the kidney (Grundemann *et al.* 1994), there is little doubt that they are protecting the organism against toxic compounds by preventing their passage to the brain or by removing them from the organism, respectively. In the case of bacterial proteins such as the *Bacillus* BmrI and *E. coli* EmrA or Mar proteins, whose expression is regulated by multiple xenobiotics (Ahmed *et al.* 1994; Lomovskaya *et al.* 1995), it also seems reasonable that they have still a major role in the protection of the cell as judged from their regulation. Yet, in other cases, it seems that given proteins have evolved to perform specific roles other than multidrug resistance, such as in the case of vesicular neurotransmitter transporters (Schuldiner *et al.* 1995), lipid translocators (Ruetz and Gros, 1995) and bacterial amino acid and sugar transporters from the ABC family (Doige and Ames, 1993). Whatever their role, multidrug transporters are interesting proteins which can handle the recognition and transport of a wide variety of toxicants. A

basic question, shared by all known transporters, pumps and channels, remains unanswered and it will probably take many years to answer: what is the molecular mechanism mediating substrate recognition and translocation? In addition, in the case of multidrug transporters, what is the basis for recognition of a wide variety of substrates with high affinity?

EmrE, because of its size and its unique properties, may provide an excellent experimental paradigm to approach the above questions.

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