Precious Things Come in Little Packages

Shimon Schuldiner*, Dorit Granot, Sonia Steiner Mordoch, Shira Ninio, Dvir Rotem, Michael Soskin and Hagit Yerushalmi

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

Abstract

The 110-amino acid multidrug transporter from E. coli, EmrE, is a member of the family of MiniTexan or Smr drug transporters. EmrE can transport acriflavine, ethidium bromide, tetraphenylphosphonium (TPP⁺), benzalkonium and several other drugs with relatively high affinities. EmrE is an H⁺/drug antiporter, utilizing the proton electrochemical gradient generated across the bacterial cytoplasmic membrane by exchanging two protons with one substrate molecule. The EmrE multidrug transporter is unique in its small size and hydrophobic nature. Hydrophobic analysis of the EmrE sequence predicts four α-helical transmembrane segments. This model is experimentally supported by FTIR studies that confirm the high α-helicity of the protein and by high-resolution heteronuclear NMR analysis of the protein structure. The TMS of EmrE are tightly packed in the membrane without any continuous aqueous domain, as was shown by Cysteine scanning experiments. These results suggest the existence of a hydrophobic pathway through which the substrates are translocated. EmrE is functional as a homo-oligomer as suggested by several lines of evidence, including co-reconstitution experiments of wild-type protein with inactive mutants in which negative dominance has been observed. EmrE has only one membrane embedded charged residue, Glu-14, that is conserved in more than fifty homologous proteins and it is a simple model system to study the role of carboxylic residues in ion-coupled transporters. We have used mutagenesis and chemical modification to show that Glu-14 is part of the substrate-binding site. Its role in proton binding and translocation was shown by a study of the effect of pH on ligand binding, uptake, efflux and exchange reactions. We conclude that Glu-14 is an essential part of a binding site, common to substrates and protons. The occupancy of this site is mutually exclusive and provides the basis of the simplest coupling of two fluxes. Because of some of its properties and its size, EmrE provides a unique system to understand mechanisms of substrate recognition and translocation.

Introduction

Multidrug transporters (MDTs) recognize a broad range of substrates with relatively high affinity and actively remove them away from the cytoplasm. Since at times the substrates are toxic, the transporters have been associated with resistance to the effect of multiple drugs, antibiotics and antineoplastic agents (Gottesman and Pastan, 1993; Nikaido, 1994). While providing an efficient survival strategy for the cell in toxic environments, the resistance associated with the activity of MDTs poses a serious problem in the clinics and agriculture. Because of the clinical relevance of these proteins and because of their apparently paradoxical ability of high affinity multidrug recognition, MDTs have been the topic of extensive studies.

Based on their primary amino acid sequence similarity MDTs have been grouped in several families (Griffith et al., 1992; Marger and Saier, 1993; Paulsen et al., 1996b; Schuldiner et al., 1995). One of them is the family of MiniTEXANs or Smr. The proteins in this family are the smallest multidrug transporters, about 100 amino acid long, that extrude various drugs in exchange with protons, thereby rendering bacteria resistant to these compounds (Grunius et al., 1992; Paulsen et al., 1996a). More than 50 genes coding for MiniTexans have been identified in several bacteria based either on their ability to confer resistance to several drugs or on amino acid sequence similarities (Paulsen et al., 1996a) (Figure 1). All the genes identified thus far are restricted to the eubacterial kingdom, both in gram negative and positive organisms. Smrs have been identified in many pathogens and they are also present in R plasmids. It has been suggested that horizontal gene transfer between bacteria is the main mechanism by which drug resistance is acquired (Davies, 1994; Rowe-Magnus and Mazel, 1999). Indeed, many of the homologue genes are found within integron regions in the different bacterial genomes, and as many as six different homologues can be found in a single genome.

Recently, the genes from Mycoplasma tuberculosis, Bordetella pertussis, Yersinia pestis, B. stearothermophilus and Pseudomonas aeruginosa have been cloned, expressed in E. coli and characterized (S. Ninio and S. Schuldiner, unpublished observations). In E. coli, four different homologues have been identified (Greener et al., 1993 and unpublished observations). However, no function has yet been assigned to the others even though they have been cloned and purified (D. Rotem and S. Schuldiner, unpublished observations). Recent interesting findings suggest the possibility that in certain organisms, some of the homologues may be functional as heterooligomers (Jack et al., 2000; Masaoka et al., 2000).

Abbreviations:

TMS - transmembrane segments
TPP⁺ – tetraphenylphosphonium
DCCD - dicyclohexylcarbodiimide
MDR - Multidrug resistance transporter
SCAM - Scanning Cysteine Accessibility Method
EmrE-mycc-His – EmrE tagged with the myc epitope and six His residues (Muth and Schuldiner, 2000)

*For correspondence. Tel. 972-2-6585992; Fax. 972-2-5634625.

© 2001 Horizon Scientific Press
Further Reading

- **MALDI-TOF Mass Spectrometry in Microbiology**
  Edited by: M Kostrzewa, S Schubert (2016)
  www.caister.com/malditof

- **Aspergillus and Penicillium in the Post-genomic Era**
  Edited by: RP Vries, IB Gelber, MR Andersen (2016)
  www.caister.com/aspergillus2

- **The Bacteriocins: Current Knowledge and Future Prospects**
  Edited by: RL Dorfl, SM Roy, MA Riley (2016)
  www.caister.com/bacteriocins

- **Omics in Plant Disease Resistance**
  Edited by: V Bhadauria (2016)
  www.caister.com/opdr

- **Acidophiles: Life in Extremely Acidic Environments**
  Edited by: R Quatrini, DB Johnson (2016)
  www.caister.com/acidophiles

- **Climate Change and Microbial Ecology: Current Research and Future Trends**
  Edited by: J Marxsen (2016)
  www.caister.com/climate

- **Biofilms in Bioremediation: Current Research and Emerging Technologies**
  Edited by: G Lear (2016)
  www.caister.com/biorem

- **Microalgae: Current Research and Applications**
  Edited by: MN Tsialoglou (2016)
  www.caister.com/microalgae

- **Gas Plasma Sterilization in Microbiology: Theory, Applications, Pitfalls and New Perspectives**
  Edited by: H Shintani, A Sadako (2016)
  www.caister.com/gasplasma

- **Virus Evolution: Current Research and Future Directions**
  Edited by: SC Weaver, M Denison, M Roossinck, et al. (2016)
  www.caister.com/virusevol

- **Arboviruses: Molecular Biology, Evolution and Control**
  Edited by: N Vasilakis, DJ Gubler (2016)
  www.caister.com/arbo

- **Shigella: Molecular and Cellular Biology**
  Edited by: WD Picking, WL Picking (2016)
  www.caister.com/shigella

- **Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment**
  Edited by: AM Romani, H Guasch, MD Balaguer (2016)
  www.caister.com/aquaticbiofilms

- **Alphaviruses: Current Biology**
  Edited by: S Mahalingam, L Herrero, B Herring (2016)
  www.caister.com/alphah

- **Thermophilic Microorganisms**
  Edited by: F Li (2015)
  www.caister.com/thermophile

- **Flow Cytometry in Microbiology: Technology and Applications**
  Edited by: MG Wilkinson (2015)
  www.caister.com/flow

- **Probiotics and Prebiotics: Current Research and Future Trends**
  Edited by: K Venema, AP Carmo (2015)
  www.caister.com/probiotics

- **Epigenetics: Current Research and Emerging Trends**
  Edited by: BP Chadwick (2015)
  www.caister.com/epigenetics2015

- **Corynebacterium glutamicum: From Systems Biology to Biotechnological Applications**
  Edited by: A Burkovski (2015)
  www.caister.com/cory2

- **Advanced Vaccine Research Methods for the Decade of Vaccines**
  Edited by: F Bagnoli, R Rappuoli (2015)
  www.caister.com/vaccines

- **Antifungals: From Genomics to Resistance and the Development of Novel Agents**
  Edited by: AT Coste, P Vandeputte (2015)
  www.caister.com/antifungals

- **Bacteria-Plant Interactions: Advanced Research and Future Trends**
  www.caister.com/bacteria-plant

- **Aeromonas**
  Edited by: J Graf (2015)
  www.caister.com/aeromonas

- **Antibiotics: Current Innovations and Future Trends**
  Edited by: S Sánchez, AL Demain (2015)
  www.caister.com/antibiotics

- **Leishmania: Current Biology and Control**
  Edited by: S Adak, R Datta (2015)
  www.caister.com/leish2

- **Acanthamoeba: Biology and Pathogenesis (2nd edition)**
  Author: NA Khan (2015)
  www.caister.com/acanthamoeba2

- **Microarrays: Current Technology, Innovations and Applications**
  Edited by: Z He (2014)
  www.caister.com/microarrays2

- **Metagenomics of the Microbial Nitrogen Cycle: Theory, Methods and Applications**
  Edited by: D Marco (2014)
  www.caister.com/n2

Order from caister.com/order
One of the smr genes, _emrE_ or _mvrC_, is an _E. coli_ gene which 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. An hydropathic analysis of the sequence reveals the presence of four putative transmembrane segments (TMS) with only one charged residue (Glu) in the putative transmembrane domain and a total of eight charged amino acids throughout the protein (Figure 1).

EmrE (Yerushalmi, 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⁺/cation antiport in proteoliposomes reconstituted with purified transporter and behave as multi-drug transporters capable of recognizing a wide range of substrates and inhibitors. The tagged transporters, flagged-Smr (Grinius and Goldberg, 1994) and EmrE-myc-His (Muth and Schuldiner, 2000), have been purified after detergent extraction and chromatography in an affinity column. Detergent solubilized EmrE was assayed by its ability to bind tetraphenyl phosphonium (TPP⁺), a high affinity ligand (Muth and Schuldiner, 2000). Reconstitution was achieved by detergent dilution and the proteoliposomes were assayed by either methyltriphenyl phosphonium (MTPP⁺), ethidium bromide or methyl viologen uptake (Grinius and Goldberg, 1994) (Muth and Schuldiner, 2000). EmrE has been purified also by taking advantage of its unique solubility in organic solvents (Schuldiner et al., 1997; Yerushalmi, 1995). After solubilization and purification, it retains its ability to transport as judged from the fact that it can be reconstituted in a functional form. EmrE transports a variety of organic cations, including methyl viologen, ethidium, acriflavine, benzalkonium and 1-methyl-4-phenyl pyridinium (MPP⁺) (Schuldiner et al., 1997; Yerushalmi, 1995).

EmrE mediated transport is driven by a proton electrochemical gradient both in intact cells and in proteoliposomes (Yerushalmi, 1995). Since the transporter functions in the intact cell also at high pH, where the sole driving force is the transmembrane electrical potential (Padan et al., 1981), it has been suggested that the exchange is electrogenic and that the stoichiometry is 2H⁺/cation (Grinius and Goldberg, 1994; Paulsen et al., 1996a; Yerushalmi, 1995).

Because of its size and solubility properties, EmrE provides a unique model for the study structure-function aspects of transport reactions in ion-coupled processes.

**Secondary Structure**

Hydrophobicity analysis of the sequence of EmrE yielded four putative transmembrane domains of similar sizes (Yerushalmi, 1995). Results from transmission FTIR measurements agree remarkably well with this notion and yielded α-helical estimates of 78 % and 80 % for EmrE in CHCl₃:MeOH and DMPC, respectively (Arkin et al., 1996). The fact that the protein retains its secondary structure in a solution of CHCl₃:MeOH fits the studies performed with subunit c of the H⁺-ATPase (Girvin and Fillingame, 1993; Girvin and Fillingame, 1995) and bacteriorhodopsin (Orekhov et al., 1994) which have been documented to retain secondary structure in this solvent mixture.

Furthermore, in the DMPC bilayer, most of the amide groups in the protein do not undergo amide-proton H/D exchange, implying that most of the residues are embedded in lipid. In addition, EmrE has been studied by high resolution NMR. A preliminary analysis of the secondary structure based on sequential NOE (nuclear Overhauser effect) connectivities, deviation of chemical shifts from...
random coil values and $^3$J(H$^N$H$^P$)-coupling constants supports a model where the protein forms four $\alpha$-helices (Schwaiger et al., 1998).

**Oligomeric Structure of EmrE**

Several lines of evidence suggest that EmrE is functional as an oligomer. The effect of several inactive mutants on the activity of the wild type protein was tested 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 significantly decrease 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. In addition, when co-reconstituted with wild type protein, they inhibit the activity of the latter in a dose dependent form up to full inhibition. We assume that this inhibition is due to the formation of mixed oligomers in which the presence of one non-functional subunit causes full inactivation. A binomial inhibition. We assume that this inhibition is due to the co-reconstituted with wild type protein, they inhibit the activity of the latter in a dose dependent form up to full inhibition. We assume that this inhibition is due to the formation of mixed oligomers in which the presence of one non-functional subunit causes full inactivation. A binomial

**The Substrate Pathway: A “Hydrophobic Channel”**

We have used the Scanning Cysteine Accessibility Method (SCAM) to assess the exposure of residues at various positions in the protein. SCAM is based on the generation of mutants in which unique reactive Cys residues are implanted at desired positions. Each mutant protein is then challenged with various sulfhydryl reagents to assess the exposure and reactivity of the Cys residue. SCAM was successfully used to identify residues exposed to the aqueous translocation pathway of ligand-activated channels and the CFTR chloride channel (Akabas et al., 1994a; Akabas et al., 1994b; Xu and Akabas, 1993). The results with EmrE show that while the residues in putative hydrophilic loops readily react, none of the residues in putative transmembrane domains is accessible to NEM, a well-characterized sulfhydryl reagent (Steiner Mordoch et al., 1999).

*Cysteine scanning:* Only the functional mutants were challenged with sulfhydryls and therefore this aspect will be discussed first. It became apparent that a very small number of residues appear essential to EmrE function. Single Cys replacements have been generated in more than 50 residues, about 50% of the protein, and nearly all of them show a measurable activity (Steiner Mordoch et al., 1999) and unpublished observations). In addition, seven out of the eight charged residues in the protein can be replaced without loss of activity (Yerushalmi and Schuldiner, 2000). Interestingly, even residues conserved in the family can be replaced without loss of activity suggesting that the conservation may be necessary for functions other than the ones that were tested. In three of the inactive mutants (Y40C, F44C and L93C) the protein is not detectable at measurable levels and, therefore, little can be concluded about the role of these residues on catalytic activity. It is possible that they play a role in folding, insertion or stability of the protein. Glu-14, the only charged residue in the putative membrane domain, cannot be replaced even with Asp, suggesting a central role in the catalytic cycle ((Grinius and Goldberg, 1994; Muth and Schuldiner, 2000; Yerushalmi et al., 1996; Yerushalmi and Schuldiner, 2000b and see also below). Analysis of 40 homologues of EmrE indicates that in TMS1 the conservation pattern shows a very clear helical periodicity. Accordingly, the consensus sequence in TMS1 is (W, Y, F) XX (L) XX (A) (i, g) XX (E, a) (V, l) XX (t, s). Cys replacements of some of the conserved residues, Ala10, Ile11 and Thr18 and also of a less conserved one, Gly17, yielded inactive mutants. Since the above residues in TMS1 are in the same face of the helix they may be playing a central role in substrate recognition or in stabilization of the negative charge (Glu-14) in the membrane. Their role needs to be studied further by a more detailed characterization of the mutant proteins and by replacements with other amino acids. In addition to the above residues, only two others, Tyr60 and Trp63 in TMS3, have been previously shown to be important for activity. Even substitution with other aromatic amino acids caused a complete loss of activity (Grinius and Goldberg, 1994; Yerushalmi et al., 1996). The finding that most residues of ion-coupled transporters can be replaced without serious impairment of function has already been described and discussed in extensive studies of the E. coli lac permease (Frillingos et al., 1998). In these studies, only six positions (out of 417) are clearly irreplaceable. It has been suggested that the role of most of the non-essential amino acids may be to provide a structural scaffold.

In our studies, mutagenesis of two groups of residues has generated proteins with modified specificity to at least...
one of the substrates of EmrE (Steiner Mordoch et al., 1999). Practically all replacements in TMS 3 display decreased resistance to acriflavine. In TMS 2, eight mutants show a significant decreased resistance to methyl viologen. They seem to cluster on two faces of the α-helix: one group 36, 47 and 54; the other one 34, 41, 48 and 52 with 42 somewhat close to this cluster. These mutants provide an excellent tool for further investigation of the location of the binding contacts of substrates.

Cysteine Accessibility Scanning: Our results demonstrate that all the residues in putative membrane domains of EmrE are practically inaccessible to 14C-NEM (Steiner Mordoch et al., 1999). The reaction of maleimides with sulfhydryl groups involves addition of mercaptide ions in the protein to the olefinic double bond (Means and Feeney, 1971). Therefore, low levels of labeling may result from either one of the following reasons or a combination of them: steric hindrance, lack of ionization of the sulfhydryl group or low reactivity because of neighboring residues. NEM is a relatively hydrophobic and small maleimide that can freely cross lipid membranes. The latter contention is supported in our work by the fact that residues in all the hydrophilic loops are fully accessible. In loops 1 (residues T28 to S33, Figure 1) and 2 (Y53 to G57), all the residues react with NEM. The lower reactivity of some of the residues in hydrophilic loops (such as at the position of L30) may reflect steric hindrance, since clearly its neighbors react quantitatively. Residues at positions P32 and S33 and Y53 to G56 may be at the edge of the membrane domain. In each, loop 3 and at the C-terminus, one residue was tested to be fully accessible to NEM.

In contrast, none of the residues in putative transmembrane domains reacted with NEM to a significant degree. In this domain, some of the sulfhydryl residues face the lipid milieu and they do not seem to be able to release their proton and therefore cannot react with NEM. As for the rest of the residues, very tight packing of the helices would prevent even relatively small reagents such as NEM from approaching.

In the case of other proteins such as the Tn-10 encoded tetracycline/H+ antiporter and the erythrocyte anion exchanger, evidence has been provided that residues in some transmembrane domains are inaccessible to the permeant sulfhydryl reagent NEM (Kimura et al., 1996; Kimura et al., 1998; Tang et al., 1998). It was therefore suggested that NEM could be used to identify the membrane embedded domains. However, other studies have clearly identified reactive residues in membrane domains (Frillingos et al., 1998; Kimura-Someya et al., 1998; Yan and Maloney, 1993; Yan and Maloney, 1995). The reactive residues are thought to delineate water filled cavities that may represent part of the substrate translocation pathway. In the extensive studies performed with the E. coli lac permease parts of the membrane embedded areas were found inaccessible to NEM. Yet, many others reacted freely with NEM (Frillingos et al., 1998). Indeed, both in the lac permease (Frillingos et al., 1998) and in UhpT (Yan and Maloney, 1995) NEM-reactive residues were found near the binding site of the hydrophilic substrates. Unlike in the other transporters, in EmrE, every single TMS was shown to be inaccessible to NEM, suggesting that the substrates are not translocated through a hydrophilic pathway.

The results with NEM cannot exclude the presence of a highly selective filter that prevents molecules other than substrates from accessing a putative aqueous pathway in EmrE. However, this possibility is refuted by FTIR studies of EmrE in which we found that a large fraction of the amide protons do not readily exchange with solvent deuterium (Arkin et al., 1996). Taken as a whole, the results described support the model of a tightly packed four helix antiparallel bundle in which the majority of the protein is well embedded in the membrane and not accessible to solvent. The boundaries of the embedded segments as estimated with this technique are in remarkable good agreement with the secondary structure determined from the NMR analysis of the protein (Schwaiger et al., 1998).

In the case of EmrE, the substrates are quite hydrophobic and therefore it may be energetically more favorable to interact directly with the protein rather than permeate through a water filled pathway. Movement of substrates through a tightly packed protein must require disruption and reorganization of the existing structure. These types of interaction have been observed in several cases. For example, NMR shows that the singular structures of soluble synthetic four helical bundles adopt a disordered array of states upon binding a hydrophobic heme cofactor. The interaction of the heme with the polypeptide is quite specific and displays a relatively high affinity in the nanomolar range (Mulholland et al., 1998). Tetraphenylphosphonium (TPP+), a high affinity substrate of EmrE, interacts specifically also with BmrR, a transcription activator of the Bmr gene, a B. subtilis multidrug transporter (Ahmed et al., 1994). In this protein, the TPP+ binds to a hydrophobic pocket with a key electrostatic component (Glu-134) at its bottom (Zheleznova et al., 1999). The entrance of TPP+ to the binding pocket occurs after unfolding of a nine-residue α-
helix. We can only speculate that a similar type of binding site may exist in EmrE as well, where only one essential negative charge (Glu-14) is present in the putative transmembrane domains of the protein. The results discussed above suggest very tight packing of the protein without any continuous aqueous domain. Therefore, entrance of the ligand to the binding site may require movement of parts of the protein. In striking contrast with the findings for the other ion-coupled transporters, the results with EmrE suggest the existence of a hydrophobic pathway through which the substrates are translocated.

A Model for Coupling of H+ and Substrate Fluxes Based on “Time-Sharing” of a Common Binding Site

In EmrE, there are eight charged residues (Figure 1); five basic (Lys-22, Arg-29, Arg-82, Arg-106 and His-110) and three acidic (Glu-14, Glu-25 and Asp-84). Seven of them are located in the hydrophilic loops and can be replaced without significant decrease in the resistance phenotype. Most of the mutations that conserve charge (Glu - Asp and Lys - Arg interconversion) have a minor effect on uptake activity, measured with the purified protein reconstituted in proteoliposomes. Only one conservative mutation (K22R) decreases uptake significantly, interestingly, this residue is the only basic residue fully conserved in EmrE. In general, replacements with Cys have lower activities than the corresponding conservative ones (Yerushalmi and Schuldiner, 2000b). Glu-14 is the only charged residue in the putative membrane domain of EmrE. Mutation in this residue has a dramatic effect on transport activity and resistance conferred by the protein. This residue is conserved throughout the fifty members of the family and it was shown to be important also for the resistance phenotype and transport activity in Smr, the Staphylococcus aureus homologue of EmrE (Grinius and Goldberg, 1994). Substitution of the corresponding residue in Smr, Glu-13, to either Asp or Gln eliminated most of the resistance to both ethidium and benzalkonium during the latter reactions are driven solely by the substrate electrochemical gradient, the rise in activity is consistent with the increasing ability of EmrE to bind substrate and release protons. The drop of activity at higher pH values is explained by the fact that release of substrate is also dependent on protonation of the binding site. The ΔpH (acid inside)-driven uptake shows steep external pH dependence as well, increasing from undetectable values below pH 7.5 to a maximum at pH 9.5 (Figure 2) (Yerushalmi and Schuldiner, 2000b). The acidic pH inside the proteoliposomes enables proper release of the substrate and proton binding. Notably, the apparent “pK” of the reactions, in which there is no transmembrane pH gradient (i.e., binding, efflux and exchange) is lower than that of the ΔpH driven uptake (Figure 2). We suggest that this is because Glu-14 senses an average pH between the acidic interior of the proteoliposomes and the medium.
Mutant proteins in which the carboxyls other than Glu-14 are manipulated either individually (E25C, E25D, D84C and D84E) or simultaneously (E25C/D84C) display the same pH dependence of uptake as wild type between pH 7.5 and 9.5 (Yerushalmi and Schuldiner, 2000b; and unpublished observations). Therefore, we conclude that Glu-14 is the major determinant of the pH dependence. When Glu-14 is replaced with Asp, binding and release of the substrate are independent of pH (Muth and Schuldiner, 2000). In other words, coupling between protons and substrate is lost and therefore accumulation of substrate at the expense of a proton gradient cannot be achieved. As expected, E14D catalyzes downhill efflux and exchange of substrate in a pH independent mode above pH 6.5 (Yerushalmi and Schuldiner, 2000b). This, again, is in line with our findings that in the E14D protein, substrate binding and release above pH 6.2, do not involve changes in the protonation state of the carboxylic residue.

We suggest that Glu-14 is an essential part of the binding domain shared by substrates and protons. Our results also indicate that occupancy of the binding domain is mutually exclusive. This fact provides the molecular basis for the obligatory exchange catalyzed by EmrE. In our view of the alternate access model for EmrE, we postulate that TPP+ is bound in a hydrophobic pocket via an interaction with Glu-14 (Yerushalmi and Schuldiner, 2000a). Glu-14 residues in each monomer presumably participate in the binding, forming a charged trimeric cluster in which one negative charge is shared and two charges are neutralized by protons. The assumption that the cluster has one negative charge is not essential for the model. However, the permanent negative charge in the binding site may serve to enhance the interaction with the positively charged substrates. The binding interaction of the substrate with different parts of the protein and the electrostatic interactions with the Glu-cluster influence the latter in such a way that induces release of the protons. Following this, the binding site, now occupied by the substrate, becomes modified so that it is accessible to the other face of the membrane. The interaction of the delocalized charge in the substrate with the three negative charges in the protein is likely to be strong in the hydrophobic environment of the putative binding site. Such a stable complex can be efficiently dissociated only when renewed proton binding to the cluster occurs. This assumption is experimentally supported by the finding that low pH accelerates TPP+ release from the protein. Therefore, we suggest that the ternary complex H+EmrE-substrate is very short lived. After protonation and substrate release, the binding site relaxes back to the other face of the membrane so that a new cycle can start.

The role of carboxylic residues in substrate binding and H+-translocation has been postulated in the mechanism proposed for the lac permease. (Frillingos et al., 1998; Sahin-Toth et al., 2000). A common feature of both transporters is that carboxyl residues with unusually high pKs play central role. Changes in the occupancy of the substrate-binding site induce changes in the protein that modifies the pKa of one or more of these residues. This results in protonation or deprotonation of the residues followed by conformational changes enabling vectorial proton translocation. Residues with an unusual high pKa are found also in other membrane proteins, such as bacteriorhodopsin and the F0F1-ATPase subunit c (Lanyi, 1999; Rastogi and Girvin, 1999). Both Asp-96, in bacteriorhodopsin and Asp-61, in subunit c, exhibit very high pKa and are critical for proton translocation in these proteins.

Several major differences exist between lac permease and EmrE besides their size: in the lac permease movement of the substrate and the coupling ion is in the same direction (co-transport or symport) while in EmrE it is in opposite directions (antiport). In addition, the lac permease has been shown to be very flexible probably contains water filled cavities, while this is not the case for EmrE. Finally, the nature of the substrate, the H+substrate stoichiometry and the specificity clearly differ. In the lac permease, substrate exchange can occur without H+ release because sugar is released prior to protons. In EmrE, on the other hand, both binding and release of substrate can occur only upon the corresponding release or binding of protons. For EmrE, these findings suggest a direct mechanism of coupling based on the mutually exclusive occupancy of a single binding site. In the lac permease, the two sites are suggested to be distinct and they interact with each other through conformational changes of the protein. While EmrE shows the simplest mode of coupling and demonstrates the advantage of this transporter as a model system, it is most likely that in the larger transporters, the more complex modes of coupling have evolved to provide additional flexibility, modes of regulation and functions still unknown to us.

**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 of plant pathogens is of major economic importance. However, these traits existed for aeons long before the drugs and their effects on patients were discovered. Resistance to a wide range of toxic 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 survive with a wide variety of compounds have been able to develop resistance. One of the strategies that evolved is removal of toxic substances by multidrug transporters. A question commonly asked about multidrug transporters is what is their “real” function (e.g see Neyfakh, 1997). Are these proteins functioning solely for protection of the organism against toxic compounds or do they have a very specific function and just accidentally, they happen to be also polyspecific. The answer seems to be a complex one: cleanly, in proteins such as Mdr1a, functioning in the blood brain barrier (Schinkel et al., 1995) or in the kidney (Grundemann et al., 1994) there is little doubt that they are protecting the organism against toxic compounds by removing them from the organism or by preventing their passage to the brain. In the case of bacterial proteins such
as the Bacillus Bmr 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 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 is the case of vesicular neurotransmitter transporters, lipid translocators (Ruetz and Gros, 1995) and bacterial amino acid and sugar transporters from the ABC family (Doige and Ames, 1993).

The function of a given transporter in survival of a particular organism in a toxic environment maybe difficult to document at times because of the large diversity of transporters with overlapping specificities.

The molecular mechanism of substrate recognition and translocation in membrane proteins and the basis for recognition of a wide variety of substrates by multidrug transporters are still unrevealed. We believe that EmrE, because of its size and its unique properties, provides an excellent experimental paradigm to approach the above questions.

Acknowledgments

Work in the author’s laboratory is supported by grants from the Deutsche-Israel Science Foundation.

References


