Minireview

A common binding site for substrates and protons in EmrE, an ion-coupled multidrug transporter

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Abstract EmrE is an *Escherichia coli* 12-kDa multidrug transporter, which confers resistance to a variety of toxic cations by removing them from the cell interior in exchange with two protons. EmrE has only one membrane-embedded charged residue, Glu-14, that is conserved in more than 50 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.

Key words: Ion-coupled transporter; Carboxylic residue; Substrate binding pocket; Proton translocation; Coupling; EmrE

1. Introduction

Transporters are responsible for creating and maintaining the different composition of the cell interior relative to the exterior in both prokaryotic and eukaryotic cells. This is also the case for the solute gradients across internal organelles such as mitochondria, synaptic vesicles and lysosomes. Some of these transporters utilize ATP as an energy source while others use ion electrochemical gradients to actively transport different substrates [1–3].

In some cases malfunction of a transporter results in diseases such as Bartter's and Gitelman's syndromes of inherited hypokalemic alkalosis [4]. Other transporters are the targets of therapeutic treatment: the antidepressant Prozac is a blocker of the brain serotonin transporter. On the other hand, some transporters prevent drug therapy: multidrug transporters that export antineoplastic agents from cancer cells and others responsible for removal of antibiotics from microorganisms [2,5,6].

An increasing number of membrane protein structures, at atomic resolution, are now becoming available, although none of them are ion-coupled transporters. Crystallization of transporters is difficult since they are in a detergent solution. In addition, it has been suggested that transporters exist in multiple conformations needed for proper translocation of substrates, a detrimental property for crystal formation. Alternative and complementing approaches are constantly being developed to circumvent the lack of structural information and to obtain sets of structural constraints that will allow an educated guess on the structure–function relationship of membrane transporters [7,8].

Charged residues are found in transmembrane domains of ion-coupled transporters, even though their insertion is energetically unfavorable. Those residues are probably transferred into the bilayer as uncharged, polar molecules, after protonation or deprotonation [9]. Also salt bridges between close enough positive and negative residues can decrease the energy required for insertion [10]. Two residues can react to form a salt bridge if they are located one turn away in the same helix, or in different helices which are close enough in the three-dimensional structure. In some transporters, such as the lac permease [11] or VMAT2 [12], some of the ion pairs were suggested to be important for transport activity or substrate recognition. Because of their location, charged residues in transmembrane domains are suggested to be important for structure or activity. In cases where substrates are charged or polar, acidic or basic residues might play important roles in the binding site of these substrates.

A clear role of charged residues in proton transfer has been demonstrated in several H^+ pumps including the light-driven bacteriorhodopsin [13], subunit c of ATPase [14] and redox-driven respiratory and photosynthetic complexes [15]. The energy input usually brings about a change in the protein which modifies the pK_a of one or more residues. This results in protonation or deprotonation of the residue, followed by several conformational changes enabling vectorial proton translocation.

A summary of the available information on the role of carboxylic residues in several ion-coupled transporters is presented in Table 1.

As can be seen in Table 1, the position of the important carboxylic groups varies in different transporters, in some of

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Abbreviations: TM, transmembrane segment; TPP^+, tetraphenylphosphonium; DCCD, dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; The mutants are named as follows: single amino acid replacements are named with the letter of the original amino acid, then its position in the protein and the letter of the new amino acid. The mutant in which two carboxyl residues are replaced with Cys is named E25C-D84C

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Table 1
The role of carboxylic residues in ion-coupled transporters

Transporter	Source	Transporter function	Length (aa), number of TMs	Carboxyl position	General comments	Reference
Residues involved in substrate binding:						
MdfA	*E. coli*	Multidrug H^+-coupled transport	410 residues, 12 TMs	Glu-26, TM 1	Part of the binding pocket of positively charged substrates	[22,23]
VMAT2, vesicular monoamine transporter 2	rat	H^+-dependent accumulation of monoamines in intracellular organelles	515 residues, 12 TMs	Asp-33, TM 1	Involved in substrate recognition. Conserved in vesicular neurotransmitters and transporters	[24]
DAT, dopamine transporter	rat	Na^+-dependent reuptake of dopamine from the synapse	619 residues, 12 TMs	Asp-79, TM 1	Involved in substrate recognition. Conserved in catecholamine transporters	[25,26]
Tet(B), Tn*10*-encoded tetracycline/H^+ antiporter	*E. coli*	Active efflux of tetracycline out of the cell	401 residues, 12 TMs	Asp-15, TM 1; Asp-84, TM 3	Both residues are part of the tetracycline binding pocket. The Tet(A) to Tet(E) efflux proteins have Asp residues in TMs 1 and 3	[27]
rOCT1	rat	First step of hepatic and renal cation excretion	556 residues, 12 TMs	Glu-475, TM 11	Conserved in the OCT-type electrogenic cation transporters	[28]
QacA	*S. aureus*	Multidrug H^+-coupled transport.	514 residues, 14 TMs	Asp-323, TM 10	Important for divalent organic cation recognition	[29]
Residues involved in the binding and translocation of co-transported cation:						
MelB, melibiose carrier	*E. coli*	Cation–sugar co-transport	469 residues, 12 TMs	Asp-51, Asp-55, Asp-120, TMs 2, 4	The hydrophobic amino-terminal of the transporter seems to accommodate the cation binding site or part of it	[30–32]
MIT, *myo*-inositol transporter	*Leishmania donovani*	*myo*-Inositol/proton symport	547 residues, 12 TMs	Asp-19, TM 1	Important for proton coupling, conserved in five inositol and sugar H^+-coupled symporters, from bacteria yeast and protozoa	[33]
NhaA, Na^+/H^+ antiporter	*E. coli*	Transport of Na^+ and H^+ in opposite directions across cytoplasmic membranes	388 residues, 12 TMs	Asp-133, TM 4	The residue plays a role in sodium binding. Conserved in transporters from bacteria and yeast	A. Rothman, unpublished observations, [34,35]
GLT-1, glutamate transporter	rat	Na^+,K^+ coupled glutamate transport into the cell	573 residues, 8 TMs	Asp-404, TM 7	Critical for interaction between K^+ and the transporter, conserved among all cloned mammalian glutamate transporters	[36]
NaDC-1, Na^+/dicarboxylate co-transporter	rabbit	Absorption of Krebs cycle intermediates by kidney and intestine	593 residues, 11 TMs	Asp-373, TM 8; Glu-475, TM 9	Both residues are important for cation selectivity	[37]
VMAT1, vesicular monoamine transporter 1	rat	H^+-dependent accumulation of monoamines in intracellular organelles	521 residues, 12 TMs	Asp-404, TM 10; Asp-431, TM 11	Both residues are important for translocation of the second proton, after substrate binding	[38]
Residues involved in either substrate binding or cation binding and translocation:						
Lactose permease	*E. coli*	H^+-coupled symport of lactose or other galactosides	417 residues, 12 TMs	Glu-325, TM 10	The only residue that appears to be directly involved in proton translocation	[7,39–41]
				Glu-126, TM 4	Part of the substrate binding site	
				Glu-269, TM 8	Important for conformational changes after lactose binding	

The table includes only examples in which the role of the residues was demonstrated in either substrate recognition or partial steps of the transport cycle.

them the residues are found in the amino-terminal half, in others in the carboxy-terminal half. The data also suggest that in several ion-coupled transporters carboxylic residues are involved in the binding of positively charged substrates, whereas in other transporters carboxyls are involved in binding and translocation of the co-transported cation. In the lactose permease, one of the best characterized ion-coupled transporters, two carboxylic residues are important for substrate binding and a different one is essential for proton binding and coupling. A simpler model system, EmrE, reveals a unique property: a single membrane-embedded charged carboxyl is part of a common binding site for substrates and protons.

2. Obligatory 'time-sharing' of a binding site common to protons and substrates provides the molecular basis of coupling in EmrE

EmrE is a member of the SMR family. This family includes small multidrug transporters widespread in the eubacterial

kingdom [16]. They are about 100 residues long, with four putative transmembrane helices. EmrE was shown to be a proton-coupled multidrug transporter [17], which functions as an oligomer, most likely a trimer [18,19].

In EmrE there are eight charged residues: 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 these residues are located in the hydrophilic loops and can be replaced without a 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. In general, replacements with Cys have lower activities than the corresponding conservative ones [20]. 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 50 members of the family and was shown to be important for the resistance phenotype and transport activity in Smr, the *Staphylococcus aureus* homologue of EmrE [21]. Substitution of the corresponding residue in Smr, Glu-13, to either Asp or Gln eliminated most of the resistance to both ethidium and benzalkonium and significantly decreased the efflux of tetraphenylphosphonium (TPP^+) out of the cell.

In EmrE, a role for Glu-14 in substrate binding was examined also by chemical modification with carbodiimides [20]. Dicylcohexylcarbodiimide (DCCD), a carbodiimide that is known to react in hydrophobic environments, inhibits uptake by wild type EmrE. In contrast, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), a water-soluble carbodiimide, causes only slight inhibition even at 40-fold higher concentrations. Since Glu-14 is the only carboxylic residue in the membrane domain and the mutant proteins, E25C and D84C, are inhibited in the same concentration range as the wild type protein, it is suggested that Glu-14 is the site of action of DCCD.

Detergent-solubilized EmrE binds TPP^+ with high affinity ($K_D = 10$ nM). TPP^+ binding to wild type EmrE is inhibited by DCCD in a dose-dependent manner (H. Yerushalmi, unpublished observations). Addition of substrates such as ethidium, acriflavine and benzalkonium during the incubation with DCCD quantitatively prevents this inactivation. We suggest that substrate protection of Glu-14 reflects the location of this residue in or near the binding site and is most likely due to steric hindrance. A striking demonstration that Glu-14 is indeed central for activity is provided also by the studies of a mutant protein with Glu-14 as a single carboxyl (EmrE E25C-D84C). This protein is capable of conferring resistance to the toxicants tested and, after solubilization, binds TPP^+ with properties similar to the wild type protein. DCCD inhibits this binding in a substrate-protectable manner in this mutant as well, confirming that Glu-14, the only carboxyl in the mutant, is the site of action of DCCD. Furthermore, the properties of Glu-14 replacements reveal that the negative charge in residue 14 is crucial for substrate recognition: the E14C mutant fails to bind substrate while the E14D mutant binds to wild type levels ($K_D = 35$ nM) [19]. The results described above corroborate the concept that the carboxyl at position 14 is part of the substrate binding domain.

To examine the involvement of Glu-14 in proton binding and translocation, individual steps of the catalytic cycle were explored. This was achieved by testing the effect of pH on TPP^+ binding to the detergent-solubilized protein [19] and on the uptake, efflux and exchange reactions [20]. TPP^+ binding to wild type EmrE increases dramatically between pH 6.5 and 8.5, suggesting that deprotonation of the site is required for substrate binding. On the other hand, binding to the E14D mutant is pH-independent in this range and decreases only at pHs below 6. These data suggest that shortening Glu to Asp decreases the pK_a of the residue to around 5, compared to the pK_a of about 7.5 in the wild type. As expected from these findings, the release of TPP^+ from the detergent-solubilized wild type EmrE is also affected by pH. It is stimulated at acidic pH, indicating that protonation of the binding domain is required for substrate release. Again, the E14D mutant shows intermediate rates of release that are pH-independent in the ranges above pH 6.4, supporting the contention that the pK_a of the Asp at position 14 is lower than 6.

The ΔpH (acid inside)-driven uptake shows steep pH dependence as well, increasing from undetectable values below pH 7.5 to a maximum at pH 9.5 [20]. This is consistent with the increasing ability of EmrE to bind substrate and release protons at the outer surface of the proteoliposomes. The acidic pH inside the proteoliposomes enables proper release of the substrate and proton binding. The fact that mutant proteins E25C, E25D, D84C and D84E display the same pH dependence as wild type supports the conclusion that Glu-14 is the residue involved in proton and substrate binding and release. Similar pH dependence is observed for downhill efflux and exchange. Even though these reactions are driven solely by the substrate electrochemical gradient, binding and release of substrate are still dependent on deprotonation and protonation of the binding site, respectively.

When Glu-14 is replaced with Asp, uptake is dramatically decreased. Because of the lower pK_a of Asp-14, binding of substrate is possible at acidic pHs. However, at the higher pH range (above 6.2), binding and release of the substrate are independent of pH. 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, at a maximal rate of about 30–50% of that of the wild type protein. In addition, and unlike the case for the wild type protein, efflux and exchange rates are already maximal at pH 6.5 and stable thereafter. 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. The 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 alternative access model for EmrE, we postulate that TPP^+ is bound in a hydrophobic pocket via an interaction with Glu-14 (Fig. 1). We assume that the Glu-14 residue in each monomer participates in the binding, forming a charged trimeric cluster, in which one negative charge is shared and two charges are neutralized by protons. The permanent negative charge in the binding site would also serve to enhance the interaction with the positively charged substrates. The interaction of the substrate with the protein (binding energy) and the cluster (maybe also electrostatic) would influence the latter

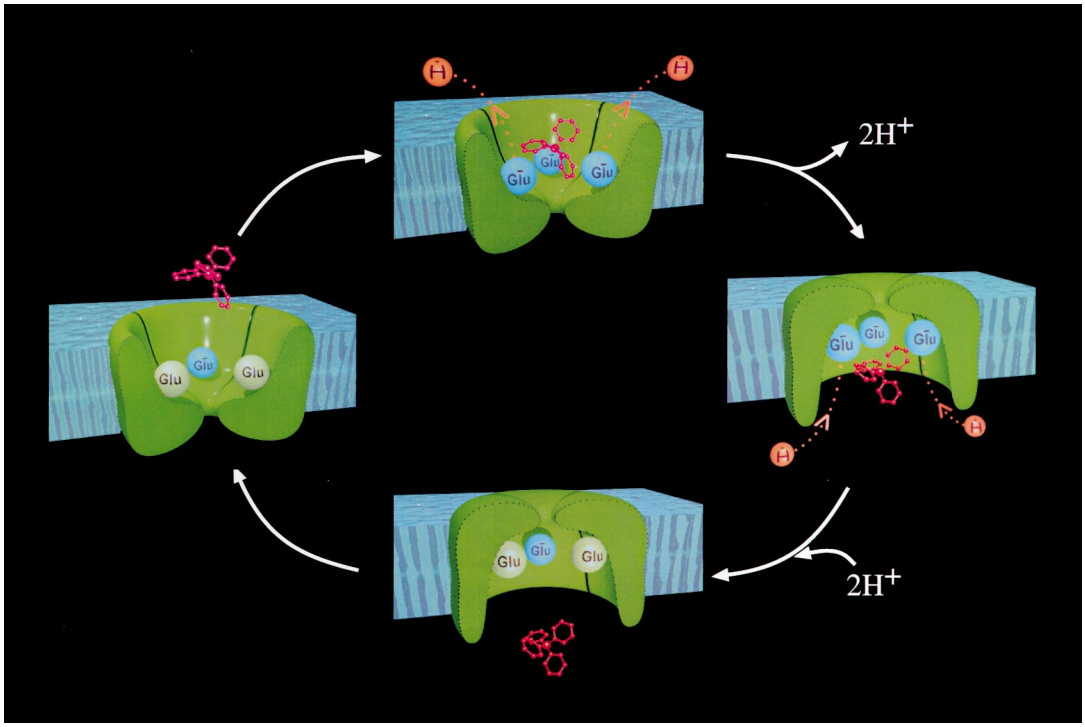

Fig. 1. Proposed mechanism for transport by EmrE. As the substrate approaches the hydrophobic binding pocket (left part), two protons are released from the negatively charged glutamate cluster (upper part). When occupied by the substrate, the binding pocket becomes accessible to the other face of the membrane (right part). The subsequent protonation of the carboxyl at position 14 allows the release of the bound substrate (lower part). The protonated binding pocket relaxes back to the original membrane face so a new cycle can start.

in a way that might induce 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. 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 central role of acidic residues in membrane domains has also been observed in many other ion-coupled transporters (Table 1). Their role in substrate binding and H^+ translocation has been postulated in a detailed mechanism proposed for the lac permease [7]. The substrate, in this case β-galactosides such as lactose, binds to a domain formed by Glu-126 and other residues. Binding to this site depends on the protonation state of Glu-325, another carboxylic acid, with an unusually high pK_a. Lactose binding induces a series of conformational changes that bring about release of protons from Glu-325. Several major differences exist between the latter model and the one for EmrE, postulated here. 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. 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. Therefore EmrE shows the simplest mode of coupling and demonstrates the advantage of this transporter as a model system.

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