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A Model for Coupling of H^+ and Substrate Fluxes Based on "Time-Sharing" of a Common Binding Site†

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ABSTRACT: Both prokaryotic and eukaryotic cells contain an array of membrane transport systems maintaining the cellular homeostasis. Some of them (primary pumps) derive energy from redox reactions, ATP hydrolysis, or light absorption, whereas others (ion-coupled transporters) utilize ion electrochemical gradients for active transport. Remarkable progress has been made in understanding the molecular mechanism of coupling in some of these systems. In many cases carboxylic residues are essential for either binding or coupling. Here we suggest a model for the molecular mechanism of coupling in EmrE, an *Escherichia coli* 12-kDa multidrug transporter. EmrE confers resistance to a variety of toxic cations by removing them from the cell interior in exchange for two protons. EmrE has only one membrane-embedded charged residue, Glu-14, which is conserved in more than 50 homologous proteins. 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. The studies suggest that Glu-14 is an essential part of a binding site, which is common to substrates and protons. The occupancy of this site by H^+ and substrate is mutually exclusive and provides the basis of the simplest coupling for two fluxes.

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 derive their energy directly from either chemical, redox, or light reactions while others use ion electrochemical gradients to actively transport different substrates (*1*−*3*).

Malfunction of a transporter can result in diseases such as Bartter's and Gitelman's syndromes of inherited hypokalemic alkalosis (*4*). Other transporters are targets for therapeutic treatment—the antidepressant Prozac is a blocker of the brain serotonin transporter. On the other hand, multidrug transporters that export antineoplastic agents from cancer cells and others responsible for removal of antibiotics from microorganisms prevent drug therapy (*3*, *5*, *6*).

An increasing number of membrane protein structures at atomic resolution are now becoming available, although none of them are of ion-coupled transporters. Crystallization of transporters is difficult since they are soluble only in 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

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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 of the structure/function relationship of membrane transporters (*7*, *8*).

Charged Residues in Transmembrane Domains

Charged residues are found in transmembrane domains of ion-coupled transporters even though their insertion is energetically unfavorable. Those residues are probably inserted into the bilayer as uncharged, polar groups, after protonation or deprotonation (*9*). Salt bridges between positive and negative residues can also 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 that are close enough in the three-dimensional structure. Some of the ion pairs in several transporters were suggested to be important for transport activity or substrate recognition. Several ion pairs (Asp-237−Lys-358, Asp-240−Lys-319, and others) are found in the lactose permease of *Escherichia coli* (*11*, *12*). A single neutral substitution of one of these residues results in loss of ability to accumulate substrates, because this leaves an uncompensated charge in the transmembrane domain. The ion pair between Asp-240 and Lys-319 may be secondarily important for function (*13*), as double neutral replacement yields permease with significant though decreased activity. Exchanging the position of the two charges (Lys-240/Asp-319) abolishes activity, indicating that correct polarity of the ion pair is essential. An ion pair was also postulated in the vesicular monoamine transporter (VMAT2), between Lys-139 and Asp-427 (*14*). Substitution of one of these residues to a neutral group abolishes transport activity, whereas mutants bearing neutral groups in both of the residues show substantial transport. Exchanging the position of the charges in the ion pair changes the specificity of the transporter, suggesting that the polarity of the ion pair is not essential for activity but may have a role in substrate recognition.

Because of their location, charged residues in transmembrane domains have been suggested as 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 (*15*, *16*), subunit c of the F_oF_1 ATPase (*17*, *18*), and redox-driven respiratory and photosynthetic complexes (*19*). The energy input usually brings about a change in the protein that modifies the pK_a of one residue or more. This results in protonation or deprotonation of the residue, followed by conformational changes enabling vectorial proton translocation. Negatively charged residues in transmembrane helices have been shown to form high-affinity binding sites for two Ca^{2+} ions in the calcium ATPase of sarcoplasmic reticulum (*20*).

Membrane-Embedded Carboxylic Residues in the Substrate-Binding Domain

Carboxylic residues embedded in the membrane are important for overall transport activity in a wide range of ion-coupled transporters. For example, in the Gram-negative Tet (A) to Tet (H) transporters, three essential Asp residues are located in TMS[1] I, III, and IX (*21*, *22*). TetK and TetL transporters from Gram-positive bacteria also include three essential carboxyls (Glu residues) but in different positions—TMS I, V, and XIII (*23*). In a unique tetracycline transporter from *Clostridium perfringens*, Glu residues important for activity are found only in TM II (*24*). Carboxyls embedded in different helices in different Tet transporters may therefore serve homologous functions.

A role of carboxylic residues in substrate recognition has been documented for vesicular monoamine transporters (VMAT's). These transporters catalyze proton-dependent accumulation of monoamine neurotransmitters into synaptic vesicles and storage organelles. A conserved Asp (Asp-33) in TM I seems to play a role in substrate selectivity (*25*). In the homologous transporter (VAChT) that transports acetylcholine, rather than the monoamines, the corresponding residue, Asp-46, can be removed with no effect on transport and binding activity (*26*).

The plasma membrane dopamine transporter (DAT), responsible for reuptake of dopamine from the synapse, is structurally unrelated to VMAT (*27*). However, it also includes an essential Asp in TM I (Asp-79). This residue is conserved in other monoamine plasma membrane transporters, such as the serotonin transporter (SERT), but, again as described for VAChT, it is substituted by a Gly in the GABA and betaine transporters (*28*). Site-directed mutagenesis of Asp-79 in DAT and Asp-98 in SERT suggests that the negative charge in this position may play a role in substrate recognition (*29*, *30*). The similar role of the carboxylic residue embedded in the first TM of both DAT and VMAT correlates with the resemblance between the substrates of these transporters—catecholamines. On the other hand, the corresponding carboxyl in VAChT is not essential for activity, presumably because of the difference between VMAT's and VAChT's substrates.

Carboxylic residues seem to play a role in the binding site of bacterial multidrug transporters as well. QacA and QacB are close related multidrug H^+-coupled transporters from *Staphylococcus aureus* (*31*). QacA confers resistance to both monovalent and divalent organic cations, whereas QacB confers lower or no resistance to divalent cations (*32*). These two transporters show only three differences in their amino acid sequences, one of them in residue 323 in TM X (an Asp in QacA and a Gly in QacB). Site-directed mutagenesis studies support the contention that the negative charge of Asp-323 may interact with one of the positively charged moieties in the divalent cationic substrate.

Carboxyls were shown to be critical for substrate recognition in MdfA from *Escherichia coli*, another bacterial H^+-coupled transporter. Cells expressing MdfA from a multicopy plasmid exhibit variable drug resistance to a diverse group of cationic or zwitterionic lipophilic compounds (*33*).

[1] 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.

Replacement of Asp-26 with Glu or with Lys results in different profiles of substrate selectivity in each of the mutants (*34*, *35*). The E26D mutant confers resistance to the positively charged substrates but not to neutral chloramphenicol, whereas the E26K mutant shows the mirror image, resistance to the chloramphenicol only. These results suggest that Glu-26 forms part of the binding pocket; therefore, the negative charge may interact directly with the positive charge of the lipophilic cationic substrates. Chloramphenicol recognition, however, is not sensitive to the charge but possibly to the size or shape of the pocket around Glu-26. If this is true, then the drug-binding pocket should be either sufficiently large or flexible to accommodate the different substrates.

The nature of the interaction between the substrates and residues in the binding pocket, as well as the role of carboxyls in this pocket, can be explored reliably only by analysis of the structure at an atomic level. Since the structural analysis of transporters has not yet yielded an atomic resolution structure, Zheleznova et al. (*34*, *36*) chose to focus on BmrR, a transcriptional activator of the multidrug transporter Bmr from *Bacillus subtilis*. BmrR activates the expression of the transporter in response to binding of a number of structurally dissimilar hydrophobic cations, many of which are also substrates of the Bmr transporter (*37*). The C-terminus of BmrR (BRC), which includes an inducer binding/dimerization domain, was separately expressed and structurally analyzed at atomic level. BRC includes a hydrophobic core with a buried carboxylic residue, Glu-134. TPP^+, an inducer of BmrR, penetrates this core, where it forms a number of van der Waals and stacking interactions with surrounding hydrophobic and aromatic residues. The most important interaction of TPP^+ with the binding core is the electrostatic contact between the negatively charged Glu-134 and the positively charged inducer. Thus, Glu-134 is essential for cationic substrates selectivity, while a number of uncharged and aromatic residues impose specific requirements on the inducer size and shape. It is tempting to speculate that other multidrug binding proteins, including transporters, utilize some of these stereochemical and structural principles to bind the substrates.

The data described above illustrates the essential role of negatively charged carboxyls in the binding of cationic substrates to ion-coupled transporters. In addition, there is growing evidence for interaction between aromatic residues, i.e., Phe, Tyr, or Trp and cationic compounds (*38*). The face of an aromatic ring provides a region of negative electrostatic potential that can bind cations with considerable strength. Aromatic residues can therefore create binding sites that are in one sense polar and able to interact with cations, yet they are overall hydrophobic and more likely to be found in membrane-embedded proteins. Aromatic residues are located near the binding site of membrane-embedded receptors and channels. Six to eight aromatic residues (Trp or Tyr) are found near the agonist-binding site of the nicotinic acetylcholine receptor (nAChR). One of these residues, Trp-149 on subunit α, correlates strongly with the cation$-\pi$ binding ability of an aromatic side chain, suggesting that this residue is in contact with the charged ammonium group of acetylcholine (*39*). Other receptors, such as muscarinic acetylcholine receptor and the dopamine receptor, show the same phenomena, i.e., a cluster of conserved aromatic resides near the binding site (*40*). In K^+ channels, cation$-\pi$ interactions involving Tyr or Trp are likely to be important for ion selectivity and for binding the blocker TEA (*41*). The above data suggest that proteins might use cation$-\pi$ interactions with aromatic residues as well as electrostatic interaction with carboxylic residues for binding cationic substrates.

Carboxylic Residues in the Binding Domain of the Coupling Ion

Carboxylic residues embedded in the membrane are also involved in the binding and translocation of the cotransported cation in both prokaryotic and eukaryotic transporters. The *E. coli* melibiose carrier (*melB*) transports a variety of sugars coupled to either H^+, Na^+, or Li^+. Three membrane-embedded Asp residues in the amino-terminal half are necessary for cation binding and cation-coupled symport (*42*, *43*). Asp residues in TM II are involved in cation recognition as well as in coupling (*44*−*46*). Asp-51 is important for Na^+ or Li^+ binding, whereas Asp-55 is important for proton or Na^+ recognition. The carboxylic residues in TM II and IV (Asp-51, Asp-55, and Asp-120) may be part of a coordination network involved in cotransported cation recognition and translocation. Asp-120 is also involved in substrate recognition, suggesting that this residue participates in the interactions between the substrate-binding site and the cation-binding site (*47*).

Another member of the 12-TMS sugar transporter superfamily is the *myo*-inositol transporter (MIT) of *Leishmania donovani*. This protein catalyzes the proton-dependent symport of *myo*-inositol into the cell, where it serves as an important precursor of various inositol phospholipids, which are involved in the attachment of the parasite to the epithelium of the insect midgut (*48*). MIT includes two membrane-embedded carboxylic residues, Asp-19 in TM I and Glu-121 in TM IV. These residues are conserved in five inositol and sugar/proton symporters from yeast and bacteria (*49*). Characterization of mutants' activity and uncoupler's effects suggests that the side chain, as well as the charge in positions 19 and 121, is critical for transport. The negative charge in position 19 seems to be important also for proton coupling. It is likely that these carboxylic residues in the amino-terminal half are on the "permeation pathway" of the substrate and protons in MIT.

Two Asp residues in TMS X and XI are fully conserved in the vesicular neurotransmitter transporter family (VNT) (*50*). Site-directed mutagenesis in VMAT1 and characterization of partial transport reactions of the different mutants in these residues suggest that both Asp residues are important for a stage beyond substrate binding, probably the transfer of a second proton (*51*). This translocation is coupled to a conformational change exposing the substrate-binding site to the other face of the membrane. This contention is supported by the fact that negative charges in TMS X and XI are also essential for transport activity in the acetylcholine transporter (VAChT), despite the difference between VAChT and VMAT substrates (*26*).

A summary of the available information on the role of carboxylic residues in several ion-coupled transporters is presented in Figure 1. As can be seen in the figure, the position of the important carboxylic groups varies in different transporters; in some of them the residues are found in the

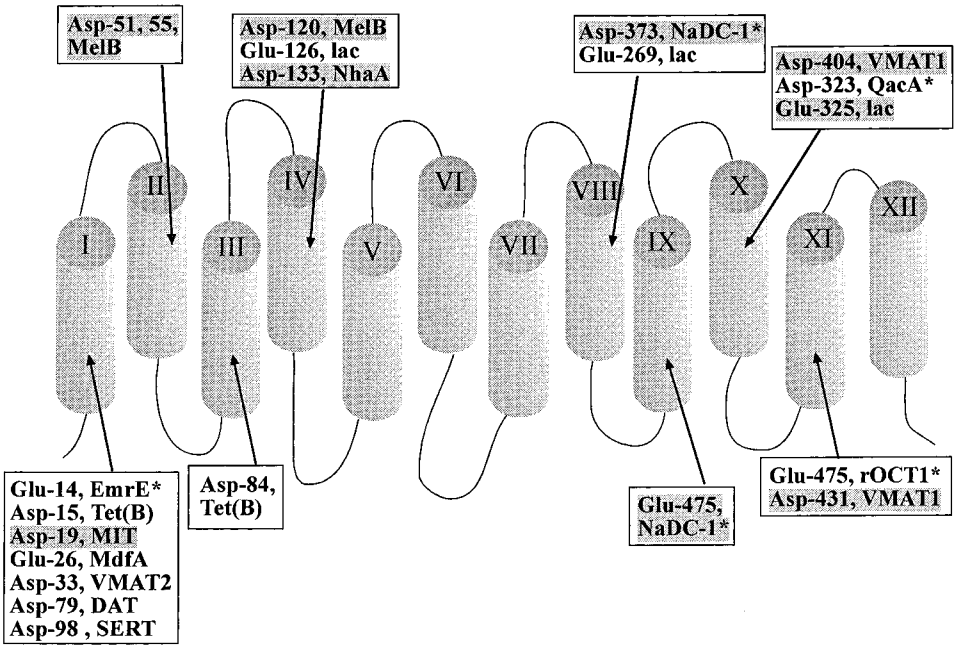

FIGURE 1: Membrane-embedded carboxylic residues with suggested roles in either substrate or coupling ion recognition. Putative transmembrane segments are shown in cylinders, connected by hydrophilic segments. Most of the transporters that are mentioned in the figure have 12 putative transmembrane segments, whereas those that have a different number of segments are marked with an asterisk (*). The residues shown in the boxes are involved in either substrate or coupling ion recognition. The latter are highlighted. For details and references see ref *69*.

amino-terminal half while in others in the carboxy-terminal half. In TM I we find the highest density of important carboxylic residues, while in helices V, VI, and VII no reports for such residues are available. 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 cotransported cation.

Carboxylic Residues in Binding Domains for Substrate and Coupling Ion

In the lactose permease, one of the best characterized ion-coupled transporters, six residues, three of them carboxyls, are important for active transport. Glu-126 (TM IV) and Arg-144 (TM V) are directly involved in substrate binding and specificity (*52*, *53*). By using site-directed spectroscopic techniques, the two residues were shown to be in close proximity in the tertiary structure of the protein, probably interacting through a salt bridge (*54*). Glu-126 and Arg-144 are part of the sugar-binding site (*55*). According to the model postulated for this binding site, charge pairing between Glu-126 and one of the amino groups of Arg-144 positions the second amino group of Arg-144 in such a manner as to allow H-bonding with the C4 and/or C3 OH of the galactosyl moiety of lactose.

In contrast, Glu-325 (TM X) is involved in H^+ translocation (*56*), and its protonation is modified by substrate binding. Glu-269 (TM VIII), Arg-302 (TM IX), and His-322 (TM X) participate in coupling H^+ and substrate translocation (*57*). In addition, Glu-269, Glu-325, Arg-302, and His-322 are in close proximity and at about the same depth in the membrane as Glu-126/Arg-144 (*7*).

The model for lactose/H^+ symport assumes that before substrate binding the permease is protonated and the H^+ is shared between His-322 and Glu-269, while Glu-325 is charge-paired with Arg-302. In this conformation, the permease binds ligand at the interface between helices IV (Glu-126) and V (Arg-144) at the outer surface of the membrane with relatively high affinity. Substrate binding induces a conformational change that leads to transfer of the H^+ from His-322/Glu-269 to Glu-325 and reorientation of the binding site to the inner surface with a decrease in affinity. Glu-325 is then deprotonated on the inside due to re-juxtaposition with Arg-302 as the conformation relaxes. The His-322/Glu-269 complex is then reprotonated from the outside surface to reinitiate the cycle (*58*).

A simpler model system, EmrE, an ion-coupled multidrug transporter, reveals a unique property; a single membrane-embedded charged carboxyl is part of a common binding site for substrates and protons.

In EmrE a Conserved, Membrane-Embedded Glutamate Is an Essential Part of the Binding Domain, Shared by Substrate and Protons

EmrE is a member of the SMR family. This family includes the smallest multidrug transporters known and is widespread in the eubacterial kingdom (*59*). They are about 100 residues long, with four putative transmembrane helices extruding various drugs in exchange with protons, thereby rendering bacteria resistant to these compounds.

EmrE is a highly hydrophobic 12-kDa protein that has been purified by taking advantage of its unique solubility in organic solvents. After solubilization and purification, it retains its ability to transport as judged from the fact that it can be reconstituted in a functional form (*60*). Hydrophobicity analysis of the sequence yielded four putative transmembrane domains of similar size (Figure 2). Results from transmission Fourier transform infrared (FTIR) measurements agree remarkably well with this hypothesis and yield α-helical estimates of 78% and 80% for EmrE in the organic

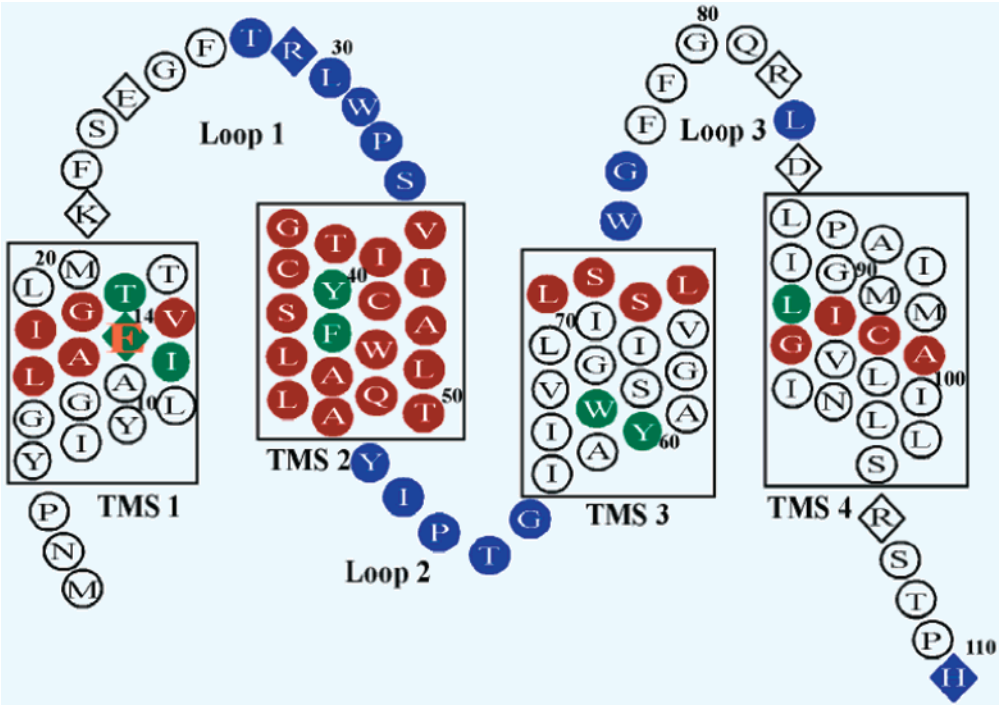

FIGURE 2: Model of the secondary structure of EmrE. The model is based on the hydropathic profile as calculated according to Engelman et al. (*9*) and experimental data (*61*−*63*). Putative transmembrane segments are shown in boxes, connected by hydrophobic segments. Each of the colored residues was replaced with Cys in a Cys-less EmrE, and the proteins were tested for activity and challenged with ^{14}C-NEM. As a result, the residues can be classified as follows: blue circles, residues accessible to ^{14}C-NEM; brown circles, residues inaccessible to ^{14}C-NEM; green circles, residues that when replaced with Cys yielded inactive proteins or the proteins were not expressed. Charged residues are shown with diamonds, and Glu-14 is shown in orange.

solvent mixture ($CHCl_3$:MeOH) and 1,2-dimyristoylphosphocholine (DMPC), respectively (*61*). 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 $^3J(H^NH^a)$ coupling constants supports a model where the protein forms four α-helices (*62*).

The transmembrane domains of EmrE are tightly packed in the membrane without any continuous aqueous domain (Figure 2), as was shown by cysteine scanning experiments and FTIR (*61*, *63*). These results suggest the existence of a hydrophobic pathway through which the substrates are translocated. The protein is functional as a homooligomer, most likely a trimer. This was shown in co-reconstitution experiments of wild-type protein with inactive mutants in which negative dominance has been observed (*64*). In addition, one high-affinity binding site has been detected per 3 mol of EmrE (*65*). Because of its size and unique properties, EmrE may provide a very useful model for understanding structure/function aspects of transport reactions in ion-coupled processes.

In EmrE there are eight charged residues (Figure 2): 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 (*66*). 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 it was shown to be important also for the resistance phenotype and transport activity in Smr, the *S. aureus* homologue of EmrE (*67*). 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 decreased significantly the efflux of TPP^+ out of the cell.

In EmrE, a role for Glu-14 in substrate binding was examined also by chemical modification with carbodiimides (*66*). DCCD, a carbodiimide that is known to react with carboxyls in hydrophobic environments, inhibits uptake by wild-type EmrE. In contrast, EDAC, a water-soluble carbodiimide, causes only slight inhibition even at 40-fold higher concentrations. In addition, it was shown that substitution of the two other carboxylic residues, Glu-25 and Asp-84, does not modify the profile of inhibition. Since Glu-14 is the only carboxylic residue in the membrane domain, it is suggested that this residue is the site of action of DCCD.

DCCD effect was also tested on binding activity of wild-type and mutated EmrE. Detergent-solubilized EmrE binds TPP^+ with high affinity (K_D = 10 nM). The binding to wild-type EmrE is inhibited by DCCD in a dose-dependent manner (H. Yerushalmi, unpublished observations). Addition of substrates such as ethidium, acriflavin, and benzalkonium during the incubation with DCCD prevents this inactivation

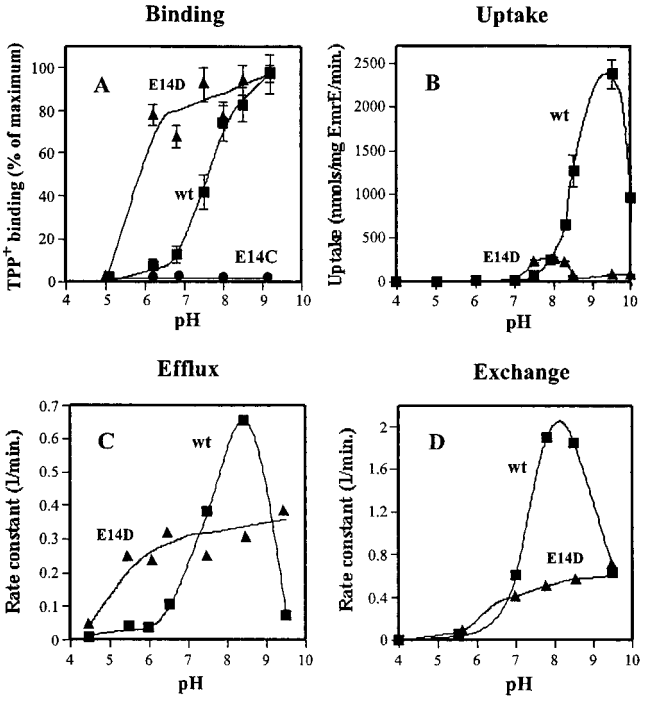

FIGURE 3: Effect of pH on binding and transport activity of EmrE. (A) TPP^+ binding to detergent-solubilized EmrE-His. Values are graphed as a percentage of maximal TPP^+ binding. Plots: purified wild-type EmrE-His (■), E14D EmrE-His (▲), and E14C EmrE-His (●). (B) Methyl viologen uptake. Ammonium-loaded proteoliposomes containing wild-type (■) or E14D mutant (▲) EmrE were diluted into an ammonium-free medium containing ^{14}C-labeled methyl viologen. Radioactivity incorporated at various time periods was measured. (C, D) Methyl viologen efflux and exchange. Proteoliposomes were loaded with ^{14}C-labeled methyl viologen; at various time points efflux and exchange of wild-type (■) or E14D mutant (▲) EmrE were measured.

in a dose-dependent manner. 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 binds TPP^+ with properties similar to those of the wild-type protein ($K_D = 42$ nM, $B_{max} = 0.21$ mol/mol). 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) (*65*). 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 (Figure 3A) (*65*) and on the uptake (Figure 3B), efflux (Figure 3C), and exchange (Figure 3D) reactions (*66*). TPP^+ binding to wild-type EmrE increases dramatically between pH 6.5 and pH 8.5, suggesting that deprotonation of the site is required for substrate binding (Figure 3A). On the other hand, binding to the E14D mutant is pH-independent in this range and decreases only at pHs below 6. These data imply that shortening Glu to Asp decreases the pK_a of the residue to around 5, compared to 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 (*65*). 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 external pH dependence as well, increasing from undetectable values below pH 7.5 to a maximum at pH 9.5 (Figure 3B) (*66*). 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. However, at pHs above 9.5 uptake activity decreases, presumably because the corresponding increase in intraliposomal pH, inhibiting protonation of EmrE. Mutant proteins E25C, E25D, D84C, and D84E display the same pH dependence as wild type between pH 7.5 and pH 9.5 (*66*). Therefore, we conclude that Glu-14 is the major determinant of the pH dependence. Similar pH dependence is observed for downhill efflux and exchange (Figure 3C,D). Activity increases dramatically between pH 6 and pH 8.5 and drops at higher pH values. 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. However, the apparent "p*K*" of these 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 3). We suggest that this is due to the fact that Glu-14 senses an average pH between the acidic interior of the proteoliposomes and the medium.

When Glu-14 is replaced with Asp, uptake is dramatically decreased (Figure 3A). Because of the lower pK_a of Asp-14, binding of substrate is possible at acidic pHs. However, efficient release on the intraliposomal side may be hindered because the pH is not acidic enough; therefore, the uptake levels do not reach their maximal value. At the higher pH range (above 6.2), however, 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 (Figure 3C,D). 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. 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

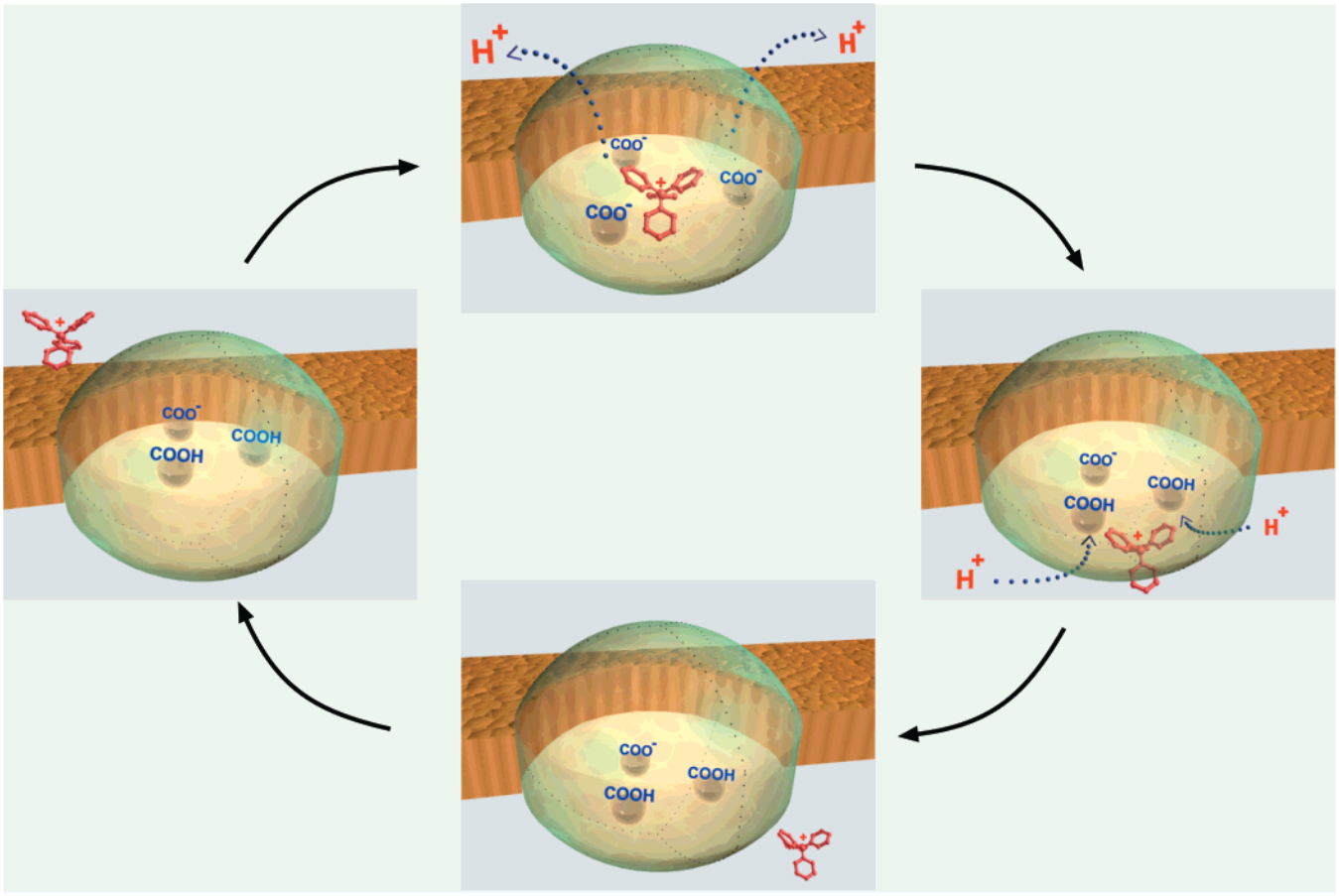

FIGURE 4: Proposed mechanism for transport by EmrE. As the substrate approaches the hydrophobic binding pocket (left), two protons are released from the negatively charged glutamate cluster (top). When occupied by the substrate, the binding pocket becomes accessible to the other face of the membrane (right). The subsequent protonation of the carboxyl at position 14 allows the release of the bound substrate (bottom). The protonated binding pocket relaxes back to the original membrane face so a new cycle can start.

alternate access model for EmrE (Figure 4), we postulate that TPP^+ is bound in a hydrophobic pocket via an interaction with Glu-14. 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 (Figure 4, left). 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 (Figure 4, top). 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 (Figure 4, right). 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 (Figure 4, bottom), 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 (*58*, *68*) and discussed above. A common feature of both transporters is that carboxyl residues with unusually high p*K*'s play central role. Changes in the occupancy of the substrate-binding site induce changes in the protein that modifies the pK_a 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 pK_a are found also in other membrane proteins, two of which were discussed earlier: bacteriorhodopsin and the F_oF_1 ATPase subunit c. Both Asp-96, in bacteriorhodopsin, and Asp-61, in subunit c, exhibit very high pK_a's 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 (cotransport or symport) while in EmrE it is in opposite directions (antiport). In addition, the lac permease has been shown to be very flexible and 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.

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