An Essential Glutamyl Residue in EmrE, a Multidrug Antiporter from Escherichia coli*

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EmrE is an Escherichia coli 12-kDa protein that confers resistance to toxic compounds, by actively removing them in exchange with protons. The protein includes eight charged residues. Seven of these residues are located in the hydrophilic loops and can be replaced with either Cys or another amino acid bearing the same charge, without impairing transport activity. Glu-14 is the only charged residue in the membrane domain and is conserved in all the proteins of the family. We show here that this residue is the site of action of dicyclohexylcarbodiimide, a carbodiimide known to act in hydrophobic environments. When Glu-14 was replaced with either Cys or Asp, resistance was abolished. Whereas the E14C mutant displays no transport activity, the E14D protein shows efflux and exchange at rates at 30–50% that of the wild type. The maximal ΔpH-driven uptake rate of E14D is only 10% that of the wild type. The mutant shows a different pH profile in all the transport modes. Our results support the notion that Glu-14 is an essential part of a binding domain shared by substrates and protons but mutually exclusive in time. This notion provides the molecular basis for the obligatory exchange catalyzed by EmrE.

Multidrug transporters (MDTs)\(^1\) 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, these transporters have been associated with resistance to the effect of multiple drugs, antibiotics, and antineoplastic agents (1, 2). 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.

MiniTEXANs or Smr, 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 (3, 4). EmrE is a MiniTEXAN from Escherichia coli. It is a highly hydrophobic 12-kDa protein that has been purified by taking advantage of its unique solubility in organic solvents (5, 6). After solubilization and purification, it 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 TMS (trans-membrane helices) of similar sizes. Results from transmission Fourier transform infrared measurements agree remarkably well with this prediction and yielded α-helical estimates of 78 and 80% for EmrE in the organic solvent mixture (CHCl\(_3\):MeOH) and 1,2-dimyristoyl phosphocholine (DMPC), respectively (7). The TMS of EmrE are tightly packed in the membrane without any continuous aqueous domain, as was shown by cysteine-scanning experiments (8). 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 co-reconstitution experiments of wild type protein with inactive mutants in which negative dominance has been observed (9). Because of some of its properties and its size, EmrE provides a unique system to identify residues involved in substrate recognition and translocation.

EmrE recognizes and translocates positively charged lipophilic compounds such as ethidium bromide, methyl viologen, and TPP\(^+\) (tetrathenylphosphonium). This fact may imply a role of charged residues in the transporter activity. In this work we analyzed the role of all the charged residues in EmrE. A total of eight residues were systematically mutagenized and only one, Glu-14, the membrane-embedded charged residue, was found to be essential.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli JM109 (10) and plasmid pKK56 (6) are used throughout this work. In plasmid pKK56, EmrE is cloned into EcoRI and HindIII sites of pKK223-3 (Amersham Pharmacia Biotech).

Mutagenesis—Mutants were obtained by polymerase chain reaction. Mutagenesis using the overlap extension procedure was described by Ho et al. (11). For most of the mutations a set of two overlapping oligonucleotide primers containing the desired mutation were constructed. The outside primers were those used for the wild type EmrE (6). The template was always wild type EmrE. In five of the mutations (E14C, E14D, R106C, R106K, and ΔH110) only two outside primers were used, and one of them contained the desired mutation.

Resistance to Toxic Compounds—For testing resistance to toxic compounds, cells were grown overnight at 37 °C in LB-Amp medium. 5

Growth was visualized after 24 h at 37 °C. The assay was repeated at least three times for each mutant.

Overexpression of EmrE—E. coli JM109 transformed with the appropriate plasmid was grown in minimal medium A supplemented with

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The abbreviations used are: MDT, multidrug transporter; TMS, transmembrane segments; TPP\(^-\), tetrathenylphosphonium; DCCD, di-cyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; MES, 4-morpholineethanesulfonic acid. 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 E29C-D84C.
MgSO$_4$ (0.01%), thiamine (2.5 μg/ml), ampicillin (0.1 mg/ml), amino acid mixture (minimum Eagle’s medium amino acids, Sigma), and glycerol (0.5%). Cells were grown at 37 °C to an A$_{600}$ of about 0.9, and isopropyl β-D-thiogalactoside was then added to a final concentration of 0.5 mM. After 2 h, cells were collected by centrifugation and washed in TSC buffer (250 mM), choline chloride (150 mM), Tris-HCl, pH 7.5 (10 mM), dithiothreitol (0.5 mM), and MgSO$_4$ (2.5 mM). At this stage the cell pellets can be kept at −70 °C until further processing. For preparation of membrane vesicles, the cells were thawed and resuspended in TSCD buffer containing DNase I.

The cells were broken up by two passages through a French press at 20,000 pounds/square inch. Undisrupted cells were removed by centrifugation at 12,000 × g for 5 min at 4 °C. The supernatant was then pelleted by centrifugation at 311,000 × g for 60 min at 4 °C. The pellet was resuspended in TSCD buffer to a concentration of about 10 mg of protein/ml and stored at −70 °C.

**Purification and Reconstitution of EmrE**—EmrE was extracted essentially as described (6), from 800 μl of membrane (8 mg of protein) with 12 ml of chloroform:methanol, 1:1. After incubation for 20 min on ice, the sample was centrifuged, and the supernatant was centrifuged. The upper phase and the interphase were removed, and the lower phase, which is enriched with EmrE, was stored at −70 °C. For analysis in SDS-polyacrylamide gel electrophoresis, a sample was dried, resuspended in sample buffer, and analyzed in 16% Tricine gels as described (12). The amount of EmrE in extracts was determined from the Coomassie-stained gels after scanning. Band intensities were measured and compared with purified EmrE standard.

For reconstitution, 6 μg of purified EmrE (in organic solvent) were mixed with E. coli phospholipids (900 μg) dissolved in 180 μl of chloroform:methanol, 1:2. The suspension was dried under argon and resuspended in 60 μl of a solution containing 190 mM NH$_4$Cl and 15 mM Tris-HCl, pH 7. The suspension was frozen and kept at −70 °C. Before the assay, the proteoliposomes suspension was thawed and sonicated in a bath type sonicator for a few seconds until clear.

**Transport Assay**—Uptake of [14C]methyl viologen into proteoliposomes was assayed by dilution of 3 μl of the ammonium chloride containing proteoliposomes (about 300 ng of EmrE) into 200 μl of an ammonium-free solution. The latter contained 37.2 μCi [14C]methyl viologen (37–48 nCi/assay), 140 mM KCl, 10 mM Tricine, 5 mM MgCl$_2$, and either 10 mM Tris (pH range 6–10) or 10 mM CAPS (pH range 10–11). At given times the reaction was stopped by dilution with 2 ml of an ice-cold solution, filtering through Schleicher & Schuell filters (0.2 μm) and washing with an additional 2 ml of solution. The radioactivity on the filters was estimated by liquid scintillation. In each experiment the values obtained in a control reaction, with 15 μM nigericin, were subtracted from all experimental points. This background was between 5 and 13% of the experimental values. The kinetics of uptake at 25 °C was measured in duplicate, at different external pH values.

For efflux, [14C]methyl viologen was added (2.1 mM) into 25 μl of ammonium chloride containing proteoliposomes (2.5 μg of EmrE) before sonication. After sonication, 2.7 μl of proteoliposomes (270 ng of the protein) were diluted into 200 μl of a solution containing 140 mM KCl, 10 mM Tricine, 5 mM MgCl$_2$, 190 mM NH$_4$Cl, and 15 μM nigericin and either 10 mM Tris (pH range 4.5–5.5) or 10 mM Tris (pH range 5.5–9.5). At given times the reaction was stopped by dilution with 2 ml of the same ice-cold solution, filtering through Schleicher & Schuell filters (0.2 μm), and washing with an additional 2 ml of solution. The radioactivity of the filters was estimated by liquid scintillation. In each experiment the values obtained after 3 h were subtracted from all experimental points. The kinetics of efflux at 15 °C was measured in duplicate at different pH values. The logarithm of the methyl viologen content at each time point was plotted versus time. The best fit was obtained by linear regression, and the rate constant was calculated for first order reaction.

The exchange assay was performed essentially as the efflux assay, except that unlabeled methyl viologen (2.5 mM) was added to the reaction solution.

**Inhibition of Uptake by DCCD and EDAC**—DCCD (Sigma) dissolved in ethanol or EDAC (Sigma) dissolved in water were added into 20 μl of ammonium chloride containing proteoliposomes to a final concentration of 2–500 μM (1:20 dilution). After 30 min at room temperature, 3 μl of proteoliposomes (300 ng EmrE) were diluted into 200 μl of an ammonium-free buffer containing 18.6 μM [14C]methyl viologen (65 nCi/assay) to determine uptake activity. Uptake of [14C]methyl viologen into the proteoliposomes was assayed as described before. Uptake at pH 8.5 was measured after 1 min in quadruplicate; the values obtained in a control reaction with 15 μM nigericin were subtracted from all experimental points.

**RESULTS**

All the Charged Residues Except Glu-14 Can Be Replaced without Impairing Resistance Phenotype—EmrE contains eight ionizable residues, three acidic (Glu-14, Glu-25, and Asp-84) and five basic (Lys-22, Arg-29, Arg-82, Arg-106, and His-110). Glu-14 and Lys-22 are conserved in more than 40 homologs of EmrE (Fig. 1, black circles).

In positions 25, 29, and 84, charged or polar residues are common (Glu, Asp, His, and Ser in position 25; Arg and Lys in position 29; and Asp, Asn, and Ser in position 84). In position 82 the following residues are found: Arg, Ser, Lys, His, and Pro. Most of the sequences available are too short to conclude about the conservation of Arg-106 and His-110. Where the sequences are available, in position 106 generally a basic residue is found, and in position 110, it is always His residue.

In this work we report the results obtained after replacing all the charged residues, each with Cys or with another amino acid bearing the same charge (Glu-Asp and Lys-Arg interconversion). In the case of His-110 the residue was deleted.

The activity of each of the mutant proteins has been tested both in *vivo* and *in vitro*. In *vivo*, the resistance conferred by each of the proteins was assessed by testing the ability of cells expressing them to grow under otherwise non-permissive conditions. This was achieved in solid media containing ethidium bromide (200 μg/ml), acriflavine (100 μg/ml), or methyl viologen (0.2 mM). Cells carrying the vector plasmid without any insert cannot grow in these media at any of the dilutions tested (Fig. 2, vector), whereas cells expressing wild type EmrE were able to grow at each of the dilutions (Fig. 2, WT). All the cells grew to a similar degree in control plates containing only ampicillin and none of the toxic compounds (data not shown). This assay provides a highly dynamic range to analyze qualitatively activity of the mutant proteins generated.

All the mutant proteins in which basic residues were mutated (K22C, K22R, R29C, R29G, R82K, R106C, R106K, and ΔHis110) were active enough to sustain a level of resistance practically identical to that of the wild type. The E25C, E25D, D84C, and D84E had a very small but significant effect on...
resistance phenotype, whereas the E25C/D84C double mutant was much less resistant as judged by this assay, especially in the presence of methyl viologen. Glu-14 is the only charged residue in the putative membrane domain and the only fully conserved acidic residue (Fig. 1). When Glu-14 was replaced with either Cys or Asp, resistance was abolished almost completely. In addition these mutants (E14C and E14D) did not grow in liquid media containing the toxins (ethidium bromide (400 μg/ml), acriflavine (50 μg/ml), data not shown).

We have demonstrated above that this assay depends on the activity of EmrE. However, no absolute quantitation can be made because the levels of expression of EmrE do differ among the various mutants. We have also previously shown (see also below) that low levels of activity are sufficient to confer resistance. Nevertheless we can conclude that, with the exception of Glu-14, each one of the charged residues can be replaced with Cys or with another amino acid bearing the same charge and produces a mutant protein that maintains an activity significant enough to confer resistance.

Most Mutants, Except E14C and E14D, Show Significant Uptake Activity after Reconstitution in Proteoliposomes—To test further the mutant proteins, they were purified and assayed for pH driven [14C]methyl viologen uptake in proteoliposomes. Except for E14C and E14D, all the single mutants display significant levels of uptake activity that range between 400 and 2900 nmol/min/mg EmrE (17–120% of wild type activity, Fig. 3). Most of the mutations that conserved charge (Glu-Asp and Lys-Arg interconversion) have a minor effect on uptake activity. The conservative mutants E25D, R82K, D84E, and R106K show more than 60% of wild type activity. His-110 can be deleted without any effect upon uptake activity. Only one conservative mutation (K22R) decreases uptake significantly, to just 22% of wild type activity. Interestingly, this residue is the only fully conserved basic residue in EmrE (Fig. 1). In general Cys replacements have lower activities than the corresponding conservative ones (Fig. 3).

Replacing both Glu-25 and Asp-84 with Cys increases the net charge of EmrE from 1 to 14. The double mutant, which confers a decreased resistance in vivo (Fig. 2), shows only marginal transport activity in proteoliposomes (103 nmols/min/mg EmrE). These results may imply the importance of net charge to EmrE activity.

Even the conservative replacement of Glu-14 with Asp, E14D, was shown to be incapable of conferring any significant resistance to toxicants. In accordance, E14C shows no uptake activity, whereas E14D displays low levels of transport (80 nmol/min/mg EmrE, Fig. 3). These results emphasize the importance of Glu-14 as the only essential charged residue in EmrE.

DCCD Inhibits Uptake by EmrE Protein—An additional way
Furthermore it was shown that E14C mutant does not bind and that the wild type uptake inhibition was tested with DCCD (●) and EDAC (△), whereas inhibition of the mutants E25C (●) and D84C (△) was tested only with DCCD.

Replacement of Glu-14 with Asp results in a protein with decreased but yet significant efflux and exchange activity (Fig. 5, B and C). As seen in Fig. 5B (efflux), about 50% of the total methyl viologen are removed from wild type proteoliposomes in 1 min. The E14D mutant extrudes methyl viologen three times slower.

The exchange reaction is 2-fold faster than the efflux, suggesting that step 6 (Fig. 5A) is rate-limiting in the latter reaction. 50% of total methyl viologen is exchanged in 30 s by the wild type protein and in 2 min by the E14D (Fig. 5C). The results shown here demonstrate that the E14D mutant binds and translocates substrate.

**E14D Shows Different pH Dependence Than the Wild Type**—E14D binds and translocates substrates downhill but does not show significant levels of ΔµH⁺-driven transport. To explore the possibility that protonation of the mutant protein is different, we tested the effect of pH on uptake, efflux, and exchange. Wild type uptake activity increases dramatically between pH 7.5 and 9.5 (Fig. 6A). Upon further increase of pH, uptake rates drop. Mutation in carboxylic groups other than Glu-14 (Glu-25 and Asp-84) does not change the pH dependence of the protein. The E25C, E25D, D84C, and D84E mutants show exactly the same pH profile as the wild type between pH 7.5 and 9.5 (Fig. 6B). These results suggest that neither one of the above residues is responsible for the pH dependence of uptake. On the other hand, E14D mutant shows drastic decrease in uptake activity, with a maximal rate that is only 10% that of the wild type (Fig. 6A). The apparent Km of this mutant to methyl viologen is 410 μM in the same range of the wild type Km (6). E14D uptake shows a shift in the optimum pH to the range 7.5 to 8.5, lower than that of the wild type protein. At other pH values uptake is practically zero.
Essential Glu Residue in EmrE

These results raise the question whether the positively charged residues in EmrE may have a role in topology determination. Unlike the case in other prokaryotic membrane proteins (14), there is no clear asymmetry in the distribution of the positive charges on both sides of the membrane. Three charged residues are located on one side of the membrane, two in the longest loop 2 and one in loop 4, whereas in the opposite face of the membrane a total of two charged residues are found, both in loop 5. The slight asymmetry can be canceled or augmented by mutation, but the effect on activity is small. We assume that if activity is maintained, the protein still displays the wild type topology. It is likely therefore that the von Heijne rule may not be directly applicable to transporters the size of EmrE.

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 EmrE. This residue is conserved in all the MiniTEXANs and was shown to be important for the resistance phenotype and transport activity in Smr, the Staphylococcus aureus homolog of EmrE (15). 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 efflux of TPP⁺ out of the cells.

An additional approach to demonstrate the central role of Glu-14 is by chemical modification with carbodiimides. DCCD, a carbodiimide that is known to react in hydrophobic environments, inhibits uptake of wild type EmrE, whereas EDAC, a water-soluble carbodiimide, inhibits only slightly even at higher concentrations. Since Glu-14 is the only carboxylic residue in the membrane domain, and the other 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. It has been shown that the reaction between DCCD and a carboxylic group requires the latter to be protonated. Interestingly, studies of the binding properties of the high affinity ligand TPP⁺ suggest that the pK of Glu-14 is above 7 (13). DCCD was shown to inhibit activity in various transporters, such as VMAT, the biogenic monoamine transporter (16), the renal Na⁺/H⁺ exchanger (17), and the renal H⁺/organic cation antiporter (18). DCCD is known to react also with subunit C in Fₒ of the H⁺-ATPase (19, 20). This protein is similar to EmrE in its size, number of charges, and the solubility in organic solvents. The site of action of DCCD is a conserved Asp or Glu in the TMS2 that is essential for H⁺ transport (21, 22). The experiments using DCCD emphasize again the importance of Glu-14 and support the contention that this residue is located in a hydrophobic environment.

A striking demonstration that Glu-14 is indeed central for activity is the fact that a mutant protein with Glu-14 as a single carboxyl (E25C/D84C) is capable of conferring resistance to the toxicants tested. In addition, after purification the activity displayed is significantly higher than the background.

More detailed information on the role of Glu-14 was obtained by exploring individual steps of the catalytic cycle. This was achieved in an independent study of the high affinity binding to the detergent-solubilized protein (13) and here by testing the effect of pH on the uptake, efflux, and exchange reactions. The negative charge in residue 14 is crucial for substrate recognition; the E14C mutant fails to bind substrate, whereas the E14D mutant binds to wild type levels. The affinity of the E14D mutant protein to TPP⁺ is 35 nM, similar to that of the wild type (10 nM) (13). In addition, the apparent Kₘ of methyl viologen uptake is 410 μM, also in the same range as the wild type Kₘ (6).

On the other hand, the pH dependence of binding differed in E14D and wild type EmrE, indicating a change in the corre-
sponding pK. The above (13) corroborate the concept that the carboxyl at position 14 is part of the binding domain. This domain cannot be occupied at the same time by protons and substrate, a phenomenon that is the basis behind the concept of coupling. Binding of substrate induces release of protons from Glu-126 (Fig. 5A, steps 1–2), substrate release from the transporter is induced by protonation of the same residue (Fig. 5A, steps 4–5).

The above contention is borne out also by the pH dependence of uphill uptake in the presence of an artificially imposed pH gradient (acid inside). The low activity below pH 7.5 is explained by the inability of EmrE to release protons and bind substrate. At the range 7.5–9.5, EmrE can 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, the decrease in uptake measured above pH 9.5 may be explained in part by the corresponding increase in the intraliposomal pH. The fact that mutant proteins E25C, E25D, D84C, and D84E display the same pH dependence as wild type support the conclusion that Glu-14 is the residue involved in proton and substrate binding and release.

When Glu-14 is replaced with Asp, uptake is dramatically decreased and is maximal at a lower pH range (7.5–8). Because of the lower pK of Asp-14, binding of substrate is now possible at a more acidic pH. 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 binding and release of the substrate are independent of pH (13). 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.

E14D does indeed catalyze downhill efflux of substrate, at a maximal rate of about 50% that of the wild type protein. As discussed above, at pH values higher than the pK, substrate binding and release are maximal and independent of pH. Indeed, efflux rate is already maximal at pH 5.5 and stable thereafter. The wild type, on the other hand, cannot catalyze efflux at pH lower than 7.0 because of its inability to bind substrate in that pH range. At pH 9.5 the rate is again lower, because of the inability of wild type protein to release the substrate (step 6, Fig. 5A).

In the exchange reaction the behavior of both proteins is very similar, except that the wild type rates for exchange are higher than for efflux, whereas the E14D mutant catalyzes both reactions at nearly the same rate. In other words, the rate-limiting step in the wild type protein is the translocation of the unloaded protonated transporter.

The central role of acidic residues in membrane domains for H+ translocation has been postulated in a mechanism proposed for the Lac permease (30). Lactose binding depends on the protonation state of Glu-325, a carboxylic acid, with an unusually high pK. Lactose binding induces a series of conformational changes that bring about release of protons from Glu-325. A major difference exists between the latter model and the one for EmrE postulated here and in Ref. 13. In the Lac permease substrate exchange can occur without H+ release because sugar is released prior to the H+. In EmrE, H+ binding is necessary for allowing release of substrate. Unlike the case for the Lac permease, we suggest that Glu-14 is an essential part of the binding domain shared by substrates and protons. The binding domain cannot be occupied at the same time by both entities. This fact provides the molecular basis for the obligatory exchange catalyzed by EmrE.

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