A Single Carboxyl Mutant of the Multidrug Transporter EmrE Is Fully Functional*

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EmrE, a multidrug transporter from *Escherichia coli* removes toxic compounds from the cell in exchange with protons. Glu-14 is the only charged residue in the putative membrane domains and is fully conserved in more than 50 homologues of the protein. This residue was shown to be an essential part of the binding site, common to protons and substrate. EmrE bearing a single carboxylic residue, Glu-14, shows uptake and binding properties similar to those of the wild type. This suggests that a small protein bearing only 110 amino acids with a single carboxyl in position 14 is the most basic structure that shows ion-coupled transport activity. The role of Glu-14 in substrate binding was examined by using dicyclohexylcarbodiimide, a hydrophobic carbodiimide that is known to react with carboxyls. Tetraphenylphosphonium binding to both wild type and the single carboxyl mutant is inhibited by dicyclohexylcarbodiimide in a dose-dependent manner. Ethidium and other substrates of EmrE prevent this inhibition with an order of potency in accord with their apparent affinities. This suggests that dicyclohexylcarbodiimide binding is sterically prevented by the substrate, supporting the contention that Glu-14, the reactive residue, is part of the substrate-binding site.

Multidrug transporters recognize a broad range of substrates with a relatively high affinity and actively remove them from the cytoplasm (1, 2). In many cases, the substrates are toxic to the cells, and their removal confers resistance. For example, multidrug transporters are responsible for resistance of cancer cells and bacteria to antineoplastic agents and antibiotics, respectively (1, 3, 4).

The SMR are the smallest multidrug transporters known (5, 6). They are about 100 amino acids long, widespread in the eubacterial kingdom. EmrE, a member of this family, is a 12-kDa transporter from *Escherichia coli* (7). This transporter is unique in terms of its size and properties and can be easily expressed and purified. Therefore, the protein can serve as a model system to study ion-coupled transporters (8).

Hydropathy analysis of EmrE predicts four α-helical transmembrane segments. Results from transmission Fourier transform infrared measurements and high resolution NMR studies agree remarkably well with this prediction (9, 10).

**Experimental Procedures**

**Bacterial Strains and Plasmids—** *E. coli* TA15 cells (19) with pT7-7 plasmid (20) containing wild type EmrE-Myc/His and pGP1-2 plasmid are used throughout all the experiments (15). The E25C/D84C mutant, in which Glu-25 and Asp-84 were replaced by Cys, was also constructed in a plasmid (20) containing wild type EmrE-Myc/His and pGP1-2 plasmid (15).

**Purification and Reconstitution of EmrE-His**—*E. coli* TA15 cells with pT7-7 EmrE-His were grown at 30 °C in minimal medium supplemented with 0.01% MgSO₄, 2.5 μg/ml thiamine, 0.5% glucose, 50 μg/ml ampicillin, and 50 μg/ml kanamycin, to an *A*₅₀₀ of about 0.8. The temperature was then increased to 42 °C to induce the T7 polymerase; 15 min later the culture was shifted back to 30 °C for 2 h. Cells were collected by centrifugation and washed in TSCD buffer (250 mM sucrose, 150 mM choline chloride, 10 mM Tris-Cl, pH 7.5, 0.5 mM dithiorethiol, and 2.5 mM MgSO₄). At this stage the cell pellets can be kept at −70 °C until further processing.

**Purification and Reconstitution of EmrE-His**—In this work we provide strong support for the role of Glu-14; a mutant bearing a single carboxylic residue (Glu-14) is shown to be active.

EmrE was shown to be a homo-oligomer by mixing of wild type and inactive mutants, both *in vivo* and *in vitro*, in which negative dominance has been observed (11). The projection structure of two-dimension crystals of EmrE revealed a non-symmetric dimeric structure as the basic oligomeric unit (12).

The protein is tightly packed, without any continuous aqueous domain (9, 13). This suggests the existence of a hydrophobic pathway in the membrane region through which the substrates are translocated.

EmrE contains eight charged residues, seven of them in the hydrophilic loops, and only one, Glu-14, is embedded in the putative membrane domain (Fig. 1). Substitution of this highly conserved residue totally abolishes resistance to EmrE substrates and dramatically affects transport activity (5, 14–16).

The results support the contention that this residue is an essential part of the binding domain shared by substrates and protons. The occupancy of this site is mutually exclusive and provides the basis of the simplest coupling for two fluxes (17).

Carboxylic residues embedded in the membrane were shown to be important for activity in various ion-coupled transporters (17, 18). In some cases, these carboxyls are involved in substrate recognition and binding, and in others, they are part of the coupled ion binding site. EmrE is unique in that a single carboxyl is involved in recognition of both substrate and the coupling ion. In this work we provide strong support for the role of Glu-14; a mutant bearing a single carboxylic residue (Glu-14) is shown to be active.
n-dodecyl-β-maltoside (DM), Anatrace, Inc. Maumee, OH, 0.5 mM phenylmethylsulfonyl fluoride, and 15 mM β-mercaptoethanol. After a 20-min incubation at 25 °C the extract was centrifuged at 435,000 × g for 20 min at 4 °C. The supernatant was incubated with the Ni-NTA beads (Qiagen, GmbH, Hilden, Germany) in the presence of 20 mM imidazole for 1 h at 4 °C and then washed with buffer containing 150 mM NaCl, 15 mM Tris-Cl, pH 7.5, 0.08% DM, 15 mM β-mercaptoethanol, and 30 mM imidazole. For reconstitution the beads bound to EmrE-His (EmrE beads) were washed first in the same buffer, with 0.08% DM and then in buffer containing 1% n-octyl-β-D-glucopyranoside (Calbiochem-Novabiochem). EmrE-His was eluted from the beads with the same buffer containing 200 mM imidazole and DM for purification or n-octyl-β-D-glucopyranoside for reconstitution.

Reconstitution was performed essentially as previously described for NhaA (21). Purified EmrE-His was mixed with a solution containing 25 mg/ml E. coli phospholipids (Avanti Inc. Alabaster, AL), 150 mM NaCl, 15 mM Tris-Cl, pH 7.5, 1% n-octyl-β-D-glucopyranoside. After sonication the mixture was diluted into NH4 buffer containing 190 mM NH4Cl, 15 mM Tris-Cl, pH 7, and 1 mM dithiothreitol. For proteoliposome formation the diluted mixture was incubated for 20 min in 25 °C and then centrifuged at 257,000 × g for 1 h. Proteoliposomes were resuspended in NH4 buffer, frozen, and kept at −70 °C. Before the assay, the proteoliposome suspension was thawed and sonicated in a bath-type sonicator for a few seconds until clear.

Uptake Assay—Uptake of [14C]methyl viologen into proteoliposomes was assayed essentially as described in Yerushalmi et al. (7). 3 µl of the ammonium chloride-containing proteoliposomes (about 600 ng of EmrE) were diluted into 200 µl of an ammonium-free solution. The latter contained 37.2 µM [14C]methyl viologen (64–74 nCi/assay), 140 mM KCl, 10 mM Tricine, 5 mM MgCl2, and 10 mM Tris-Cl, pH 8.5. At given times the reaction was stopped by dilution with 2 ml of the same ice-cold solution. The samples were filtered through Millipore filters (0.22 µm) and washed with an additional 2 ml of solution. The radioactivity on the filters was measured 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 no more than 10% of most experimental values. The kinetics of uptake was measured in duplicate at 25 °C.

Binding Assay—Tetraphenylphosphonium (TPP+) binding was assayed essentially as described in Muth and Schuldiner (15). EmrE-His membranes were solubilized with 0.8% DM in NH4 buffer (190 mM NH4Cl, 15 mM Tris-Cl, pH 7) at 25 °C for 15 min. Ni-NTA beads were washed twice in distilled H2O and once in NH4 buffer with 0.08% DM (NH4-DM buffer). The beads (10 µl per assay) were bound to EmrE-His proteoliposomes from solubilized membranes or to purified protein by incubation at 4 °C for 45 min. The unbound material was discarded, and the EmrE-His bound to beads (EmrE beads) was washed with NH4-DM buffer. After two washes with NH4-DM buffer, 200 µl of buffer containing 12.5 nM [3H]TPP+ (5 Ci/mmol, Amersham Pharmacia Biotech) were added, and the samples were incubated for 15 min at 4 °C. In each experiment the values obtained in a control reaction, with 25 µM unlabeled TPP+, were subtracted. The binding reaction was stopped by separating the beads from the supernatant by pulse centrifugation and then removing the supernatant. The bead fraction was then incubated for 10 min at room temperature with 450 µl of NH4-DM buffer containing 150 mM imidazole to release the EmrE-His and [3H]TPP+ from the beads. After spinning down the beads, the [3H]TPP+-associated radioactivity was measured by liquid scintillation. All binding reactions were performed in triplicate.

In experiments that tested the effect of EmrE substrates on [3H]TPP+ binding, the substrates were added with the [3H]TPP+ to the EmrE beads.

For examining the effect of dicyclohexylcarbodiimide (DCCD) on [3H]TPP+ binding, EmrE beads (10 µl) were incubated at 4 °C in a 100-µl suspension of NH4-DM buffer containing different concentrations of DCCD. In some of the experiments EmrE substrates (ethidium bromide, acriflavine, methyl viologen, 1-methyl-4-phenylpyridinium (MPP+), or benzalkonium) were added to the buffer as well as DCCD. [3H]TPP+ binding was assayed on the EmrE beads after the reaction was stopped by the addition of 1.4 ml of NH4-DM buffer and immediate spinning down.

When the effect of pH on TPP+ binding was tested, the solutions contained 140 mM KCl, 10 mM Tricine, 5 mM MgCl2, and 0.08% DM. The different solutions were buffered with 30 mM MES (pH range 6.5–7.5) and Tris-Cl (pH range 7.5–9.5) and titrated with either KOH or HCl. In these experiments the binding buffer contained 6 nM [3H]TPP+ (30 Ci/mmol, Amersham Pharmacia Biotech).

For measurement of the Kd calculations of E25C/D84C EmrE, 1 µg of purified protein was used. TPP+ binding was measured in a range of concentrations (3–300 nM) essentially as described in Muth and Schuldiner (15). Kd and Bmax were calculated from the Scatchard plot.

RESULTS

EmrE with a Single Carboxyl (Glu-14) Displays Properties Similar to Those of the Wild Type—In previous studies Glu-14 was shown to be central for activity. This residue is involved in binding both substrate and proton but separately in time (15, 16). To further characterize the importance of Glu-14 for EmrE activity, a single carboxyl mutant (E25C/D84C) was constructed. This mutant confers resistance to EmrE substrates, although it is less resistant compared with the wild type, especially in the presence of methyl viologen. (16). Because of the uniqueness of such a protein with a single carboxy, we tagged it with a His6 tag, purified it, and characterized its activity. After purification on a Ni-NTA column the protein was reconstituted with E. coli lipids by detergent dilution. The proteoliposomes loaded with NH4Cl were diluted into an ammonium-free buffer to generate a pH gradient (7). The E25C/D84C mutant shows significant uptake activity (50% of the wild type
activity, Fig. 2A), and transport is inhibited by the ionophore nigericin that prevents generation of a pH gradient (not shown). The pH dependence of the uptake reaction is practically identical to that of the wild type (not shown). This demonstrates again that Glu-14 is indeed central for transport activity whereas the other carboxylic residues are less important.

The single carboxyl mutant binds the high affinity substrate TPP with properties comparable with those of the wild type protein \(K_d = 28 \text{ nM}, B_{\text{max}} = 0.18 \text{ mol/mol}\). The affinity of the mutant protein is 3 times lower than that of the wild type \(K_d = 10 \text{ nM}\) (15) suggesting that the two hydrophilic carboxyls have only a minor effect on substrate binding. The total number of binding sites determined from the experiment is 0.15–0.2 mol/mol. This value is slightly lower than that of the wild type \(0.25–0.3 \text{ mol/mol}\) (15). The difference can result from the fact that the mutant protein was only partially purified, as compared with the wild type EmrE that was purified to homogeneity.

In addition, the findings indicate the presence of a residue with an apparent pK of 7.3–7.5 in the binding site of the wild type as well as the mutant.

EmrE substrates inhibit TPP binding of the E25C/D84C mutant at the same concentration range as for the wild type (Fig. 2C (15)). Benzalkonium, ethidium, and acriflavine inhibit binding with a relatively high affinity; 10 \(\mu\text{M}\) benzalkonium or 100 \(\mu\text{M}\) ethidium and acriflavine fully inhibit TPP binding. As previously shown for the detergent-solubilized wild type EmrE, the affinity for MPP is lower, and even at 10 \(\text{mM}\) it does not inhibit binding totally, whereas methyl viologen does not inhibit at all, at the concentrations tested.

Single carboxyl EmrE shows uptake and binding properties similar to those of the wild type. This similarity between the E25C/D84C mutant and the wild type protein supports the contention that Glu-14 is the only carboxyl essential for transport activity and for proton and substrate binding.

DCCD Inhibits TPP Binding of Wild Type EmrE in a Substrate-protectable Manner—Characterization of the single carboxyl mutant emphasizes the importance of this residue. The role of Glu-14 in substrate recognition and binding was further examined by chemical modification by carbodiimides. In previous studies we showed that DCCD, a carbodiimide that is known to react with carboxyls in hydrophobic environments, inhibits uptake by wild type EmrE in a dose-dependent manner (16). In addition, it was shown that substitution of each of the two other carboxylic residues, Glu-25 and Asp-84, does not modify the profile of inhibition. Glu-14 is the only carboxylic residue common to the wild type and the two other mutants; therefore it was suggested that this residue is the site of action of DCCD.

In this work the DCCD effect on binding of the high affinity substrate, TPP, was tested directly. The binding to wild type EmrE is inhibited in a time-dependent manner (Fig. 3A). The reaction with the detergent-solubilized protein can be con-
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**Fig. 3. Effect of substrates on inhibition of TPP$^+$ binding by DCCD.** EmrE-His membranes were prepared as described under "Experimental Procedures." After solubilization wild type EmrE-His (65 µg of total membrane protein/assay, A) or E25C/D84C EmrE-His (195 µg of total membrane protein/assay, C) were immobilized on Ni-NTA beads. The EmrE beads were incubated with buffer containing DCCD (500 µM) with (■) or without (▲) ethidium bromide (150 µM). After stopping the reaction and washing, [3H]TPP$^+$ binding was measured. B and D, the experiment was performed essentially as in A and C except that the EmrE beads were incubated with DCCD (500 µM for the wild type and 250 µM for the E25C/D84C mutant) in the presence of different concentrations of substrates as specified in the figure. After 90 min for the wild type and 35 min for the mutant the reaction was stopped and the samples were tested for [3H]TPP$^+$ binding. Every experiment was performed at least twice, and one representative result is shown.

trolled and manipulated very easily. To slow down the reaction it was carried out at 4 °C. Under these conditions 50% of inhibition can be seen after 25 min of incubation in 4 °C, whereas 2 h are needed for full inhibition (Fig. 3A). Addition of 150 µM ethidium, one of the EmrE substrates, during the incubation with DCCD prevents this inactivation almost completely. Lower ethidium bromide concentration has only a partial protective effect. Other substrates of EmrE prevent inhibition by DCCD as well (Fig. 3B). Very low concentrations of benzalkonium (1 µM) prevent most of the DCCD effect on binding, whereas ethidium and acriflavine protect against DCCD inhibition at higher concentrations. MPP$^+$ shows only 50% protection, even at high concentration (20 mM), whereas methyl viologen does not prevent DCCD inhibition even at concentrations as high as 100 mM. The effect of various substrates on DCCD prevention of binding is in the same range and order of potency as the inhibition of TPP$^+$ binding.

We suggest that substrate protection against the DCCD reaction is most likely due to steric hindrance, and therefore, its target residue is in or near the binding site.

**Substrates Prevent Inhibition by DCCD Also in the Single Carboxyl Mutant—**As was shown here the activity of the single carboxyl mutant E25C/D84C is comparable with that of the wild type EmrE. In addition, the only site of action for DCCD in this mutant is the single carboxylic residue, Glu-14. Therefore, this mutant can be used for further examining the role of Glu-14 in substrate recognition.

DCCD inhibits also TPP$^+$ binding to E25C/D84C EmrE in a time-dependent manner (Fig. 3C). As previously shown for the single replacement D84C (16), this mutant is also more susceptible to DCCD than the wild type. After less than 5 min of incubation with 500 µM DCCD, 50% of the binding is inhibited, whereas 25 min are needed for the same inhibition of the wild type. Substitution of the carboxylic residues in the hydrophilic loops (Glu-25 and Asp-84) seems to increase DCCD accessibility to the protein and therefore increases the sensitivity. Ethidium bromide (150 µM) prevents most of the inhibition by DCCD. Other substrates of EmrE protect against the DCCD effect in a dose-dependent manner (Fig. 3D). As described above for the wild type, the substrates with the higher affinity are benzalkonium, ethidium bromide, and acriflavine that react in the micromolar range; MPP$^+$ affinity is lower (milimolar range), and methyl viologen does not show any protection at all. Most of the substrates more efficiently prevent the inhibition of binding by DCCD in the wild type compared with the single carboxyl mutant. For example 100 µM acriflavine prevents the DCCD effect totally in the wild type, whereas in the mutant it prevents only 60% of the DCCD effect. This is in line with the observed higher rate of inhibition by DCCD in the mutant that decreases the efficiency of the protection.

The protection against the DCCD effect by substrate in the single carboxyl mutant strengthens again the suggestion that Glu-14 is an essential part of the substrate-binding site. Binding of substrate to this residue decreases the accessibility of DCCD to this site and therefore prevents its action. The substrate specificity and the concentration range for inhibition of TPP$^+$ binding and for protection against the DCCD effect is similar for the single carboxyl mutant. This confirms the above contention that the inhibition of the DCCD reaction with Glu-14 by substrates is most likely due to a steric effect.

**DISCUSSION**

EmrE has eight charged residues, three of which are carboxyls (Glu-14, Glu-25, and Asp-84, Fig. 1). Glu-14 is strictly
conserved in more than 50 homologues of EmrE and is the only charged residue essential for transport (16). Replacement with an Asp has a profound effect on the behavior of the protein, because in the mutated protein the Asp residue has a much lower pK than the corresponding Glu (15). Our results support the notion that Glu-14 is an essential part of the binding domain shared by substrates and protons but mutually exclusive in time (17).

DCCD, a carbodiimide that was shown to react specifically with Glu-14, was previously shown to inhibit uptake of methyl viologen by proteoliposomes (16). In this work we tested the effect of DCCD directly on binding of the high affinity substrate, TPP⁺, to the detergent-solubilized transporter. The detergent-solubilized protein maintains its ability to recognize most substrates at high affinity; the pK of Glu-14 is not modified, and the sensitivity to DCCD is conserved. The inhibition of transport by DCCD is observed at lower concentrations, but this may reflect the partition of this hydrophobic compound in the membrane, reaching an effective concentration much higher than in the bulk. The binding assay allows for a very simple termination of the reaction by dilution and washing as opposed to the proteoliposomes system where the DCCD partitions into the lipid phase and is not removed. DCCD inhibits binding of TPP⁺ to EmrE in a time-dependent manner, and this is prevented by the presence of EmrE substrates (benzalkonium, ethidium, acriflavine, and partially by MPP⁺). The different substrates inhibit TPP⁺ binding and the DCCD effect in the same range of concentrations, indicating that the binding affinity of each substrate is comparable with its potency to prevent the DCCD effect on binding. Our explanation for this effect is that binding of substrate limits the accessibility of DCCD to its site of action, Glu-14, suggesting that this residue is part of the substrate binding site.

In the single carboxyl mutant (E25C/D84C) the substrate effect on inhibition by DCCD is comparable with what was shown for the wild type. In this mutant, the carboxylic residue Glu-14 is the only site of action for DCCD. Therefore, preventing this reaction between DCCD and Glu-14 by substrates strongly strengthens the role of Glu-14 in the substrate-binding site.

Carboxylic residues embedded in the transmembrane region were shown to be part of the substrate- or coupling ion-binding site in a range of larger transporters that are more complex than EmrE (reviewed in Refs. 17 and 18)). In many ion-coupled transporters this carboxyl is found in the first transmembrane segment. This is the case for MdfA, another E. coli multidrug transporter (22), the Tet transporter Tet(B) (23, 24), and eukaryotic systems as well. In the vesicular and plasma membrane monoamine transporters, an Asp in transmembrane segment 1 is important for substrate recognition (25–27).

EmrE and other transporters in the SMR family are the smallest ion-coupled transporters known (5, 6). This means that a simple structure of 110 amino acids, organized as an oligomer, is capable of transporting a wide variety of substrates. In this work, we show that even a simpler structure, which includes only one carboxylic residue, is active. E. coli cells expressing the single carboxyl mutant, E25C/D84C, show significant resistance to EmrE substrates (16). The purified protein reconstituted in proteoliposomes catalyzes ΔpH-driven transport of [³⁵S]methyl viologen with properties similar to those of the wild type protein. In addition, the affinity of binding as well as the number of binding sites is comparable with that of the wild type protein.

Both wild type and mutant show similar pH dependence of TPP⁺ binding. We have previously shown that this pH dependence reflects the fact that binding and release of the substrate occur only upon the corresponding release and binding of protons (15).

It is quite surprising that in such a small protein, with only eight charged residues, removal of two negative charges has relatively little effect on activity, implying that the overall structure must be maintained. In addition, the negative charges in the loop seem to play a minor role in “guiding” the positively charged substrates, because the affinity of TPP⁺ is not dramatically altered in the single carboxyl mutant. The results imply also very little, if any, effect of these two residues on insertion into the membrane and on protein stability. The single carboxyl mutant has four net positive charges. We therefore speculate that to prevent electrostatic repulsion with the cationic substrates most of the positive charges in the protein may be neutralized by ion pairing to species such as phospholipids for example.

The simplicity and size of EmrE allow for a detailed dissection of the role of Glu-14. The construction of an active transporter that has a single carboxyl residue at position 14 provides even stronger support for the previous conclusions. This mutant also gives us the opportunity to try to find the most basic structure that shows transport activity.

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