The membrane topology of EmrE – a small multidrug transporter from *Escherichia coli*

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Abstract EmrE is a multidrug transporter from *Escherichia coli* that belongs to the Smr family of small multidrug transporters. The secondary structure of EmrE consists of a four helical bundle, as judged by different techniques. EmrE has been extensively characterized; nevertheless, the membrane topology of EmrE has not been determined yet. Previous work with a homologous Smr protein provided partial information of the membrane topology, however the location of the carboxy-terminus remained inconclusive. In this work we probed the membrane topology of EmrE, focusing on the carboxy-terminus of the protein, using two independent approaches. Our results support a secondary structure where the carboxy-terminus faces the cytoplasm, while the first loop faces the periplasm.

Key words: EmrE; Membrane protein; Drug resistance; Smr; Cysteine accessibility

1. Introduction

Multidrug transporters actively remove toxic compounds from the cytoplasm of cells. They are widespread from bacteria to man and have been associated with multidrug resistance [1,2]. Numerous multidrug transporters have been identified to date and based on amino acid sequence similarity, they were classified into several protein families [3,4]. The smallest multidrug transporters belong to the Smr family. Proteins in this family are ~110 amino acids long and extrude various drugs in exchange with protons, thereby rendering bacteria resistant to these compounds [5,6].

The most extensively characterized Smr protein is EmrE, from *Escherichia coli*. Hydropathic analysis of the EmrE sequence predicts four α-helical transmembrane segments. This model is experimentally supported by Fourier transform infrared spectroscopy studies that confirm the high α-helicity of the protein and by high-resolution heteronuclear nuclear magnetic resonance analysis [7,8]. The transmembrane segments of EmrE are tightly packed in the membrane without any continuous aqueous domain, as was shown by cysteine scanning experiments [9]. These results suggest the existence of a hydrophobic pathway through which the substrates are translocated. EmrE has only one membrane-embedded charged residue, Glu-14, which is conserved throughout the Smr family, and was shown to be part of a binding site common to protons and substrates [10,11]. The occupancy of the binding site by H^+ and substrate is mutually exclusive and provides the basis of the simplest coupling for two fluxes [12,13].

When studying the mechanism of action of a membrane protein such as EmrE, it is essential to determine the membrane topology of the protein. This information cannot be deduced from structural models of the protein that are based on detergent-solubilized samples of the protein. Moreover, knowledge of the membrane topology is required in order to fully understand these structural models. While the membrane topology of EmrE has not been determined before, that of QacC, a homologue from *Staphylococcus aureus*, was investigated by construction and analysis of a series of qacC-phoA and qacC-lacZ fusions [14]. This work confirmed the basic four-helical bundle model for Smr proteins, however the data regarding the possible orientation of the carboxy-terminus were not conclusive. To clarify this point we probed the membrane topology of EmrE, focusing on the carboxy-terminus, as well as the first loop connecting transmembrane domains (TM) 1 and 2 of the protein.

2. Materials and methods

2.1. Topology studies using sulfhydryl reagents

E. coli strain HMS174 (DE3) (Novagen) and plasmid pT7-7 EmrE-His were used throughout these experiments. Single cysteine mutants of EmrE were constructed on a cysteine-less background. All EmrE mutants used in this study were previously characterized, and all are functional proteins [9,15,16]. The cells were grown at 37°C overnight in minimal medium A supplemented with $MgSO_4$ (0.01%), thiamine (2.5 μg/ml), ampicillin (0.1 mg/ml) and MEM amino acid mixture (Sigma). The next day, the cells were diluted 1:20 into the same medium without ampicillin, and incubated for an additional 2 h before expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside. Two hours later the cells were harvested and washed at room temperature with Tris–NaCl buffer (150 mM NaCl, 15 mM Tris, pH 7.5), and divided into tubes, about 10 ml of cultured cells per assay. Expression of the NhaA protein was done using the *E. coli* strain TA16 harboring the plasmid paxH with the single cysteine mutants E241C and H225C, both functional mutants described before [17].

For reaction with sulfhydryl reagents the cells were incubated with 10 mM of the reagents 2-aminoethyl methanethiosulfonate hydrobromide (MTSEA, Anatrace), or sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES, Anatrace) for 10 min at 25°C with shaking, and then washed three times with Tris–NaCl buffer. Control cells were incubated as above, without the MTS reagents. Membranes were prepared from the cells essentially as described before [18], and His-tagged protein was purified as described previously [19]. For labeling with fluorescein-5-maleimide (NEM-fluorescein, Pierce), purified protein bound to beads was washed once with Tris–NaCl buffer supplemented with 0.5% (w/v) sodium dodecyl sulfate (SDS) and 6 M urea.

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The protein was then incubated with the same buffer containing 0.5 mM NEM-fluorescein for 20 min at room temperature. To stop the reaction, it was diluted with the same buffer containing β-mercaptoethanol at a final concentration of 5 mM. The protein was then eluted from the beads using 400 mM imidazole, and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Fluorescence labeling analysis of the gel was done using a Fujifilm LAS-1000 imaging system and digitally analyzed with Image Gauge 3.46 Fujifilm software.

2.2. C-terminal orientation studies using INDIA HisProbe-HRP

Right-side-out membrane vesicles (RSO) were prepared by lysozyme–EDTA treatment and osmotic lysis as described [18]. Inside-out membrane vesicles (ISO) were prepared by passage through a French pressure cell at 4000 psi [20]. RSO or ISO membranes (~50 μg total membrane protein) from cells expressing His-tagged EmrE or His-tagged NhaA were diluted in 100 μl Tris–NaCl buffer, and incubated for 1 h at room temperature with the reagent INDIA HisProbe-HRP (Pierce) at a final concentration of 40 μg/ml. The membranes were then washed twice with the same buffer, to remove unbound probe, and centrifuged at 244 000 × *g* for 30 min. The membranes were resuspended with the same buffer to their initial volume, and dot-blotted onto nitrocellulose paper, in various dilutions. The nitrocellulose paper was then dried, and incubated with a blocking solution for 20 min. Finally, the horseradish peroxidase (HRP) substrate was added, and the signal was detected using a Fujifilm LAS-1000 imaging system. As a control, the addition of the probe was done after the membranes had been dot-blotted onto the nitrocellulose paper. Membranes from cells expressing EmrE without a His tag were used as a negative control in these experiments.

3. Results and discussion

The membrane topology of EmrE was studied using two independent approaches. The first approach was to probe, in whole cells, the accessibility of single cysteine residues engineered within a cysteine-less protein, to different sulfhydryl reagents. This was done by assaying the ability of these reagents to inhibit labeling of the protein with the fluorescent reagent NEM-fluorescein. More specifically, a set of single cysteine mutants was constructed on the background of a cysteine-less EmrE mutant, with cysteines implanted at various locations along the first loop and carboxy-terminus of the protein. Whole cells expressing these EmrE constructs were incubated with either MTSEA or MTSES, which have been shown to be membrane-permeant and -impermeant, respectively [21]. The cells were then washed to eliminate unbound reagent, membranes were prepared, and the histidine-tagged EmrE protein was purified using Ni-NTA beads. The amount of reagent bound to the protein was estimated by reacting the purified denatured protein, bound to beads, with the fluorescent sulfhydryl reagent NEM-fluorescein. Results were obtained after analysis of the protein on SDS–PAGE.

As a control, this assay was also carried out with NhaA, a protein with a known membrane topology [22]. Two single cysteine mutants of NhaA were assayed, E241C, facing the cytoplasm, and H225C, facing the periplasm. Whole cells expressing these mutants were incubated with MTS reagents. The percentage of free cysteines left after this reaction was assessed by reaction of the purified denatured protein with NEM-fluorescein. The product was analyzed on SDS–PAGE, examined for fluorescence (Fig. 1A), and labeled with Coomassie to determine total protein amounts (Fig. 1B). Residue 241 did not label with NEM-fluorescein since it fully reacted with the permeant reagent MTSEA, however the labeling after treatment with impermeant MTSES was practically identical to that of the control, without MTS treatment (Fig. 1A, left panel). Residue 225 gave a different picture, and was not labeled after treatment with either MTSEA or MTSES, as expected for a periplasm-facing residue (Fig. 1A, right panel). The gel was digitized and quantified for fluorescence levels and total protein levels. The results for the fluorescence labeling were corrected for the relative protein amounts at each experimental point. The adjusted results are summarized in a graph (Fig. 1C).

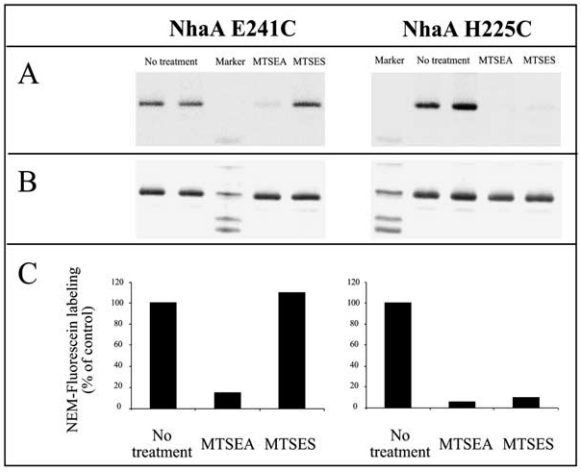

Fig. 1. Topology of NhaA using MTS reagents. Whole cells expressing NhaA single cysteine mutants E241C (left panel) and H225C (right panel) were reacted with 10 mM of the sulfhydryl reagents MTSEA or MTSES. To estimate the percentage of free cysteines left after this reaction, the proteins were purified using metal chelate chromatography and labeled with the fluorescent reagent NEM-fluorescein. A: The mutant proteins were analyzed on SDS–PAGE, and NEM-fluorescein labeling was documented. B: The same gel was also analyzed for total protein using Coomassie staining. C: The percentage of NEM-fluorescein labeling relative to a control without MTS reagents was adjusted to the relative protein level, and plotted for each mutant. NhaA protein bands were identified using molecular size markers, and were not observed when cells harboring plasmid alone were used (not shown). The experiments were performed at least twice. In each experiment, not fewer than three MTS concentrations were tested. For the sake of simplicity only one concentration is shown.

This system was then used for the analysis of EmrE constructs, with single cysteine residues engineered throughout the hydrophilic regions of the cysteine-less protein. The location of these cysteines can be viewed in a schematic representation of EmrE (Fig. 2). The results for these experiments were analyzed as before, and fluorescence was adjusted for the total protein level at each point. The results show that the first loop of EmrE, containing residues K22 and W31, is accessible to both permeant and impermeant MTS reagents and therefore NEM-fluorescein labeling of this loop was significantly inhibited (Fig. 3, right side), suggesting this loop faces the periplasm of the cell. The carboxy-terminus of EmrE, containing residues N102 and H110, reacted only with the permeant reagent MTSEA, but not with impermeant MTSES, as revealed from the NEM-fluorescein labeling pattern (Fig. 3, left side). These results suggest that the carboxy-terminus of EmrE faces the cytoplasm of the cells.

The location of the carboxy-terminus of EmrE was further verified using an independent technique. In this technique we probed the accessibility of the carboxy-terminal histidine tag of EmrE to the reagent HisProbe-HRP. This is a nickel-activated enzyme that binds to histidine tags, and catalyzes a

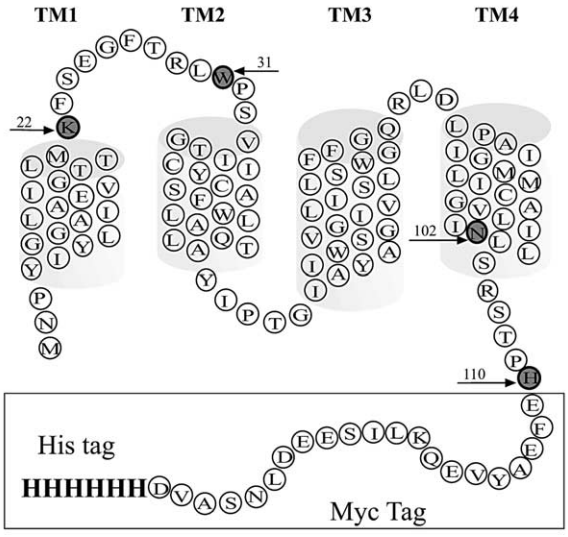

Fig. 2. Location of single cysteines implanted within the EmrE protein. Model of the secondary structure of EmrE-His, with four transmembrane regions predicted by hydropathy analysis. Residues which have been replaced, one at a time, with cysteines are highlighted with a dark background, and marked with an arrow bearing the position number. The six histidine tag and myc epitope linker are highlighted with a box.

reaction that results in light emission when given an appropriate substrate. In these experiments RSO or ISO are incubated with the reagent under native conditions. After the incubation the membranes are washed to remove unbound probe, and dot-blotted on nitrocellulose paper. Finally substrate for the HRP enzyme is added and the light reaction is monitored using digital imaging. The results show that while

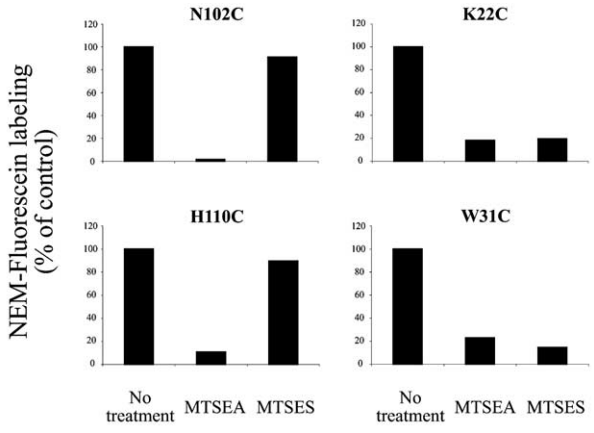

Fig. 3. Topological studies using MTS reagents. Whole cells expressing single cysteine mutants of EmrE were reacted with the sulfhydryl reagents MTSEA or MTSES. To estimate the percentage of free cysteines left after this reaction, the proteins were purified using a Ni-NTA column and labeled with the fluorescent reagent NEM-fluorescein. The mutant proteins were analyzed on SDS–PAGE, and NEM-fluorescein labeling was measured. The percentage of NEM-fluorescein labeling relative to a control without MTS reagents was adjusted to the relative protein level, and plotted for each EmrE mutant. EmrE protein bands were identified using molecular size markers, and were not observed when cells harboring plasmid alone were used (not shown). The experiments were performed at least twice. In each experiment, not fewer than three MTS concentrations were tested. For the sake of simplicity only one concentration is shown

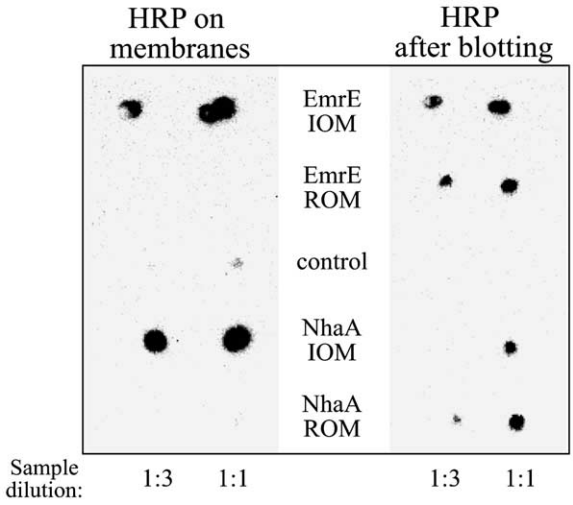

Fig. 4. The carboxy-terminus of EmrE faces the cytoplasm. RSO and ISO were prepared from cells expressing histidine-tagged EmrE or histidine-tagged NhaA. The membranes were probed with the reagent INDIA HisProbe-HRP, a nickel-activated enzyme that binds to histidine tags and catalyzes a reaction that results in light emission. The probe was incubated with the membrane vesicles before (left panel) or after (right panel) they had been blotted onto nitrocellulose paper. The HRP substrate was added to initiate the light reaction, and the blot was documented using digital imaging. Membranes expressing EmrE without a histidine tag (Control) served as a negative control.

EmrE in ISO reacts readily with the reagent (Fig. 4, left side), in RSO it reacts only when the probe is added after blotting of the membranes, when they are no longer in their native state (Fig. 4, right side). As a control, this experiment was also carried out with the membrane protein NhaA, and the results obtained (Fig. 4) were very similar to those obtained with EmrE, as expected from the known topology of NhaA [22]. Taken together, these results support a secondary structure for EmrE where the carboxy-terminus faces the inside of the cell.

We have determined the three-dimensional structure to 6.5 Å resolution of EmrE by electron cryo-microscopy of two-dimensional crystals [23]. The structure obtained was that of an asymmetric homodimer. However, at the current resolution of our map we could not directly resolve the identity of the transmembrane helices. Therefore, two possible models of EmrE were suggested, one in which the two monomers in the dimer have the same orientation in the membrane, and a model where the two monomers have opposite orientations, with inverted topologies. The results we present here clearly demonstrate that the C-termini and the first loops of all the monomers are facing the same side (cytoplasm and periplasm, respectively), and therefore it is not likely that the protein has a mixed topology.

In summary, using two independent approaches, this work demonstrates that the first loop of EmrE, connecting between TM1 and TM2, faces the periplasm of cells, while the carboxy-terminus of the protein faces the cytoplasm.

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