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Anti-parallel Membrane Topology of a Homo-dimeric Multidrug Transporter, EmrE

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EmrE in *Escherichia coli* belongs to the small multidrug resistance (SMR) transporter family. It functions as a homo-dimer, but the orientation of the two monomers in the membrane (membrane topology) is under debate. We expressed various single-cysteine EmrE mutants in *E. coli* cells lacking a major efflux transporter. Efflux from cells expressing the P55C or T56C mutant was blocked by the external application of membrane-impermeable SH-reagents. This is difficult to explain by the parallel topology configuration, because Pro55 and Thr56 are considered to be located in the cytoplasm. From both the periplasm and the cytoplasm, biotin-PE-maleimide, a bulky membrane-impermeable SH-reagent, could access the cysteine residue at the 25th position in the presence of transport substrates and at the 108th position. These observations support the anti-parallel topology in the membrane.

Key words: multidrug resistance, SMR, membrane-impermeable SH-reagent (DTNB, AMS, biotin-PE-maleimide), ethidium efflux, dual membrane topology, EbrAB.

Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2-2′disulfonic acid; AP, alkaline phosphatase; AS1, drug-hypersensitive *E. coli*; BM, biotin-PE-maleimide (N′-[2-N-maleimido)ethyl]-N–piperazinyl-D-biotin-amide hydrochloride); CL, cysteine-less mutant in which all native cysteine residues are replaced with serine; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); TPMP, triphenylmethylphosphonium.

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EmrE, one of the small multidrug resistance (SMR) transporter family, is a proton/drug anti-porter and expels various toxicants from *Escherichia coli* cells to confer the multidrug resistance (*1–3*). EmrE, whose molecular mass is as low as 12 kD, is considered to consist of four membrane-spanning helices as shown in Fig. 1. Therefore, EmrE had been considered to function as an oligomer, and at present, a homo-dimer structure is generally accepted (*3–6*). This dimeric structure is supported by the following facts: EbrAB and YkkCD from *Bacillus subtilis*, and YdgEF from *E. coli*, are composed of paired components that are encoded in distinct operons, and it is noted that the simultaneous expression of both components is absolutely required to confer the resistance (*7–11*). Thus, it is accepted that the SMR family functions as a homo- or hetero-dimer.

What is the membrane topology of these two components? It seems natural to consider the parallel topology, especially for a homo-dimeric transporter such as EmrE. In fact, several biochemical results supporting the parallel topology have been reported (*12–14*). In addition, a parallel topology was also reported for SugE, another SMR homologue in *E. coli* (*15*). On the contrary, the anti-parallel configuration of EmrE and SugE was reported (*16–18*). An investigation using an electron microscope (EM) and a modelling analysis based on the EM data have also proposed the anti-parallel topology (*4*, *19*, *20*). Thus, the membrane topology of EmrE is now open to discussion.

Previously, we investigated the membrane topology of EbrAB, a hetero-dimeric SMR in *B. subtilis*, by examining the reactivity of a membrane-impermeable SH-reagent to various engineered single-cysteine mutants, and concluded the anti-parallel configuration (*21*). For EbrA, both the *N*- and *C*-termini locate in the periplasmic space while both the *N*- and *C*-termini of EbrB locate in the cytoplasmic space. YdgEF, another hetero-dimeric SMR, was also reported to have an anti-parallel configuration (*16*). The anti-parallel configuration of EbrAB and YdgEF follows the 'positive-inside' rule stating that each protein is orientated such that the side containing the higher number of positively charged Arg and Lys residues faces the cytoplasm (*16*, *21*, *22*).

On the other hand, EmrE obviously does not have the 'positive-inside' bias (*16*, *17*), implying that we lose one of the powerful criteria determining the membrane topology. It is noteworthy here that certain mutants of EbrA or EbrB conferred the resistance to the cells when only one or the other of the EbrA or EbrB mutant was expressed. The mutants were prepared so that the 'positive-inside' bias was removed from EbrA or EbrB (*10*). In other words, a homo-dimeric 'Ebr drug-transporter' was created. Since EbrAB has the anti-parallel configuration (*21*), it is probable to consider that this 'homo-dimeric transporter' also has the anti-parallel configuration in the active form, and that EmrE, a homo-dimer, possibly has the same configuration.

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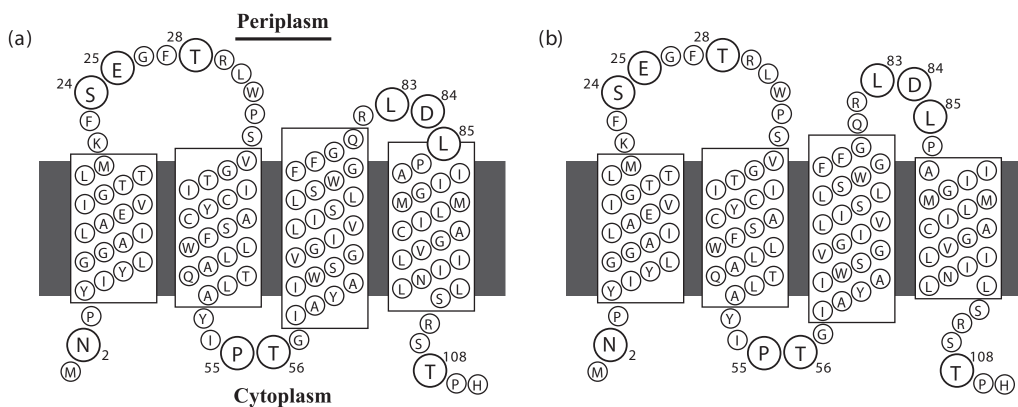

Fig. 1. **Two independent topological models of EmrE.** (a) A parallel topology model (*12–14*); (b) a model based on the proposed 3D structure from low-resolution electron microscope (*20*). In (b), terms of 'periplasm' and 'cytoplasm' are not drawn because this is assumed to be the anti-parallel topology. The large circles indicate the replaced residues. Here, Arg and Lys were not replaced so as to retain the number of positive charges.

In the present study, the membrane topology of EmrE was investigated. We were particular about using no fusion proteins, since the addition of a hydrophilic peptide may easily induce a topological constraint to SMR (*10*, *21*). Various single-cysteine mutants were engineered, which were exposed to membrane-impermeable SH-reagents from the outside of intact cells. The loss of the extrusion activity was observed for the P55C and T56C mutants. This is contradictory to the parallel membrane topology because Pro55 and Thr56 are indicated to be in the cytoplasm (Fig. 1a). Furthermore, we performed labelling experiments using the bulky membrane-impermeable SH-reagent, biotin-PE-maleimide (N′-[2-(N-maleimido)ethyl]-N-piperazinyl-D-biotinamide hydrochloride, abbreviated by BM). The cysteine residue introduced at the 108th position (T108C mutant) could be modified from both the periplasm and the cytoplasm. The cysteine residue at the 25th position (E25C) was labelled only from the cytoplasm. However, in the presence of ethidium bromide or triphenylmethylphosphonium (TPMP), transport substrates, this cysteine residue was labelled from the periplasm. These observations support the anti-parallel topology, which is sometimes called the 'dual topology' configuration.

MATERIALS AND METHODS

Chemicals—DTNB [5,5′-dithiobis-(2-nitrobenzoate)] and BM were purchased from Dojindo (Kumamoto, Japan), and AMS (4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid) was from Invitrogen-Molecular Probes, Inc. (Carlsbad, CA). All other reagents were of analytical grade from Wako Co. (Osaka, Japan). The amounts of proteins were estimated using a standard method (BCA protein Assay Reagent, Pierce Biotechnology, Rockford, IL).

Plasmids and Expression of EmrE—The expression plasmid was derived from pGEM T-easy (Promega, Madison, WI), and EmrE (GenBank M62732) was fused to the initiation codon of *lacZα*. The host cell was the E. coli strain AS1 from Dr Kawagishi of Nagoya University, Japan, and grown in LB liquid medium containing 1% bacto tryptone, 0.5% yeast extract and 0.5% NaCl supplemented by 50 μg/ml ampicillin and 100 μM isopropyl-1-thio-β-D-galactoside, IPTG. The optimal ethidium extrusion was attained by the induction of this IPTG concentration. Plasmids of various mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sets of two overlapping primers. The DNA sequences were confirmed using the standard procedure (377 DNA sequencer, Applied Biosystems, Foster City, CA). Some products were confirmed by the standard N-terminal assay after purification.

Measurement of Ethidium Efflux—The basic procedure was the same as that previously described (*10*). Cells at mid-log phase were harvested and washed with 50 mM potassium phosphate (pH 7). Ethidium bromide was loaded into cells by incubation of the cells in the buffer containing 70 μM ethidium bromide for 30 min on ice. After washout of the ethidium, the extrusion was started by the addition of 10 mM succinate, a respiratory substrate at 25°C. Ethidium inside the cells was assayed by its fluorescence. The efflux rates were estimated from the initial slope of the plot of the ethidium-remaining cells versus time.

Detection of the Labelling by BM—The intact cells were incubated in a medium containing 50 mM potassium phosphate (pH 7), 10 mM sodium succinate and 200 μM BM at room temperature for 30 min. After the washout by centrifugation, the proteins were separated by SDS PAGE (18% acrylamide gel) and transferred to a PVDF membrane (Millipore, Billerica, MA) by electrophoresis. The blotted membrane was immediately soaked in a Tween-PBS solution supplemented with 1% BSA, followed by incubation in the presence of 1/10,000-diluted Streptavidin-alkaline phosphatase (AP) (Amersham Bioscience, Buckinghamshire, UK) at 45°C overnight. The image of the Streptavidin-AP hybridization was developed by an NBT/BCIP system (Novagen, Madison, WT) after three washings of the blotted membrane with a Tween-PBS solution at 45°C.

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RESULTS

Cysteine-less (CL) and Single-cysteine Mutants have as High an Efflux Activity as the Wild Type—The wild-type EmrE has three cysteine residues in the putative transmembrane (TM) domains (Cys39 and Cys41 in TM2 and Cys95 in TM4; Fig. 1). For the construction of mutants having only a single cysteine, all three intrinsic cysteine residues were first replaced by serine residues. The CL mutant was expressed in a drug-hypersensitive *E. coli* strain, AS1, lacking AcrA, a subunit of a potent MDR transporter. The efflux activity of ethidium from the AS1 cells harbouring a vacant plasmid is much lower than the cell expressing the wild-type EmrE (data not shown). The ethidium-efflux activity from CL is compatible to that of the wild-type cells (Table 1), as Mordoch et al. reported (*23*). Starting from this CL background mutant, 10 single-Cys mutants were constructed, in which Asn2, Ser24, Glu25, Thr28, Pro55, Thr56, Leu83, Asp84, Leu85 or Thr108 was substituted by cysteine. These residues are located at putative loops connecting successive membrane helices as indicated in both topological models (Fig. 1). The single-cysteine mutant proteins were also expressed in AS1 cells, and the efflux activities were examined (Table 1). Except for P55C, all these single-cysteine mutants retained a sufficient efflux activity to be analysed, because this indicates the holding of valid higher structures. There is a possibility, on the other hand, that the low activity of P55C (ca. 60% of control) may be due to the low amount of expression. The amounts of the expression of P55C as well as the other mutants were then checked. The extraction with chloroform/methanol followed by the SDS-polyacrylamide electrophoresis analysis showed essentially no difference in the expressed protein amounts (data not shown).

Effect of Chemical Modification with DTNB or AMS, Membrane-impermeable SH-reagents, on the Efflux Activity—We first turned our attention to the relatively low activity of P55C. Proline is generally accepted to be a structure breaker; therefore, the proline residue in this loop might play an important role in forming a specific tertiary structure. Therefore, we attempted the chemical modification using SH-reagents against Cys55 (P55C) and Cys56 (T56C) locating putatively in the cytoplasm loop (Fig. 1) as well as against Cys of the other single-cysteine mutants.

We used a membrane-impermeable SH-reagent, DTNB (*24*), which was applied to *intact* cells expressing single-cysteine mutants (250 μM, 30 min). Of interest, the treatment exerted a significant effect only on P55C or T56C (Table 1). The efflux rates of the other mutants were not significantly affected by the treatment. We do not know how this was caused because the reagent did not react with the engineered cysteine residues or because the modification did not affect the transport activities except for the P55C and T56C mutants. It should be noted that Pro55 and Thr56 are located in the cytoplasm according to Fig. 1a, and that the membrane-impermeable SH-reagent reacted from the outside of the intact cells. Subsequently, we will show the evidence that Cys56 was actually modified by DTNB (Fig. 2a). This observation suggests that at least one of

Table 1. **Effects of various SH-reactive reagents on ethidium efflux activities from intact cells expressing single-cysteine mutants.**

Single-Cys mutants	Cysteine-reactive reagents			
	None (%)	DTNB[a] (%)	AMS[b] (%)	BM[c] (%)
WT	100	93 ± 5	94 ± 4	96 ± 6
ΔEmrE	2	ND	ND	ND
CL	100	98 ± 5	93 ± 5	98
N2C	78	101 ± 6	82 ± 5	92
S24C	74	81 ± 10	88 ± 7	93
E25C	88	96 ± 2	92 ± 7	103
T28C	80	90 ± 7	85 ± 2	99
P55C	60	15 ± 5	6 ± 2	10
T56C	93	10 ± 2	1 ± 2	8
L83C	98	78 ± 8	83 ± 5	97
D84C	104	83 ± 6	81 ± 3	84
L85C	94	95 ± 2	108 ± 5	ND
T108C	106	85 ± 4	80 ± 5	99

The important data are underlined.
All values are expressed as percentages relative to the cells expressing the wild-type EmrE. The evaluation method of the efflux activities is described in the text. ND means not determined.
[a]DTNB (final concentration of 250 μM) was applied to the intact cells expressing the listed mutants for 30 min, and the efflux activities were then measured. For the incubation medium, see the text. The values are mean ± S.D. for 5-independent experiments.
[b]The activities obtained after the AMS modification (2 mM and 30 min) are listed. The other experimental conditions were the same as that for DTNB.
[c]BM (200 μM, 30 min) was used, and the other experimental conditions were the same as that of DTNB.

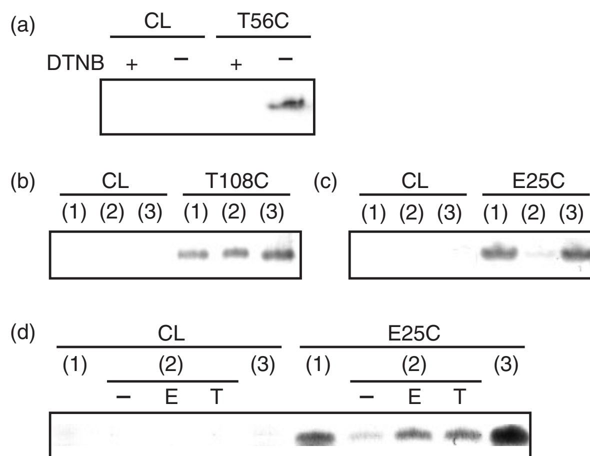

Fig. 2. **Labeling with BM of single-cysteine mutants.** (a) Labelling of T56C. The sign (+) means that before BM-labelling, DTNB (250 μM) was applied from the outside of the intact cells. The sign (−) means the results without DTNB treatment. CL means cysteine-less mutant, and for CL, no labelling is confirmed as the control. (b), (c) and (d), labelling of T108C and E25C, respectively. Details of labelling methods are described in the text. Briefly, (1) is the labelling from the cytoplasm, (2) is from the periplasm and (3) is from both sides. In (d), labelling of E25C from the periplasm (Method 2) in the presence of the substrates was performed. Here, E and T denote the ethidium (100 μM) and TPMP (150 μM), respectively.

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the Cys55 and Cys56 of the homo-dimeric EmrE mutants faces the periplasm.

This suggestion rests on the membrane impermeability of DTNB, which has been described in (*24*). We obtained the following observation that supports this notion. When we treated intact cells expressing the CL mutant with a membrane-permeable NEM, N-ethylmaleimide, (*25*) from the outside, the efflux activity was completely lost. On the other hand, the same treatment with DTNB did not abolish the activity (data not shown). These observations imply that the membrane-permeable NEM enters the cytoplasm to dissipate the membrane energization, while DTNB cannot penetrate through the cell membrane.

The suppressed ethidium efflux from the DTNB-treated intact cells was effectively restored by application of a reducing reagent, i.e. 100 μM dithiothreitol (DTT) for 30 min from the outside of the cells (data not shown). Since DTT itself did not affect the efflux of the T56C mutant, the recovery of the function was attributed to the reductive dissociation of 5-mercapto-2-nitrobenzoate, half of the DTNB that had reacted with the Cys56 residue. Essentially, the same results of DTT were obtained with P55C. Since DTT is hydrophilic, this observation is also consistent with the location of T56C and P55C deduced earlier.

Another membrane-impermeable SH-reagent, AMS (*25*), was also tested. After exposure of the intact cells to 2 mM AMS for 30 min, the ethidium extrusion from cells expressing P55C or T56C was blocked to a large extent (Table 1).

Treatment With a Bulky Membrane-impermeable SH-reagent—The earlier results suggest that at least one of Cys55 and Cys56 of the dimeric EmrE mutants faces to the periplasm, contrary to the parallel topology. However, one should consider the possibility that membrane-impermeable SH-reagents, such as DTNB or AMS, may reach the cytoplasm through a 'central pore' formed by a dimer of EmrE that react with the cysteine residues in the cytoplasm. To rule out this possibility, a bulky SH-reagent, BM, was employed that is too big to penetrate through the 'central pore'. Similar to DTNB and AMS, BM (200 μM) externally reacted with the intact cells expressing various single-cysteine mutants for 30 min, and the efflux activities were then measured. These results are shown in Table 1, demonstrating the loss of the efflux activity in P55C and T56C.

DTNB Can React With Cys56 of T56C—Since the detection of the labelled BM can be performed with a high sensitivity using Streptavidin-conjugated AP, we then tried to directly detect the labelling of BM: a separation by SDS-PAGE was performed, followed by blotting and subsequent detection with AP. A labelled EmrE was easily detected even when intact cells were used without further purification. This may be achieved, presumably because there are no other BM-modified molecules having a molecular mass around that of EmrE. As shown in Fig. 2a, the signal from T56C is clearly visible. After the treatment with DTNB, no signal of BM from T56C was visible, implying that the labelling by DTNB and BM is competitive. We, then, concluded that DTNB react to Cys56 of T56C, thereby stopping the extrusion.

Labelling with BM from Periplasm, Cytoplasm or Both Sides—The results obtained so far strongly suggest that at least one of the Cys55 and Cys56 of the dimer EmrE mutant is exposed to the periplasm. To fully understand the membrane topology, the location of other residues should be examined. The reactivity of BM against various single-cysteine mutants was then checked. Unfortunately, the labelling only with T108C and E25C was clearly visible beside T56C. The reason for this is not known, but one possible reason might be the steric hindrance of the bulky BM. Figure 2b shows the results of T108C. Here, we employed the following three different modification methods with a bulky membrane-impermeable BM:

(i) Intact cells were first treated with DTNB and then sonicated after removing DTNB, followed by exposure to BM. By this method, BM may modify the cysteine residue *facing the cytoplasm*, because the cysteine residue facing the periplasm had already been modified with DTNB.
(ii) Intact cells were exposed to BM. BM may modify the cysteine residue *facing the periplasm*.
(iii) Intact cells were suspended in a medium containing BM, and the sonicated membrane was further exposed to BM. BM may modify the cysteine residues *from both sides*.

The band densities obtained by methods 1 and 2 are weak, and that of method 3 is much denser (seemingly almost twice). The band-density ratios were not always constant among the several experiments, and the results reported here are semi-quantitative. This may imply that one of the Cys108s of the dimer is in the cytoplasm while the other Cys108 is in the periplasm.

Figure 2c shows results of E25C. Different from T108C, the band treated by method 2 was not visible or very faint in some cases, while the bands by methods 1 and 3 are visible with approximately the same densities or with the density of (*3*) being slightly denser. This might mean that Cys25 is located only in the cytoplasm, which is strange if the anti-parallel membrane topology of the dimeric EmrE may be the case. Next, in the presence of substrates, ethidium or TPMP, we performed the labelling experiment from the outside. These results are shown in Fig. 2d, where method 2 along with '–' stands for the experiment in the absence of any substances, 'E' stands for the experimental condition in the presence of ethidium (100 μM), and 'T' stands for the experimental condition in the presence of TPMP (150 μM). Interestingly, the bands of 'E' and 'T' became visible.

For T56C, we did show the modification from the periplasm (Fig. 2a). However, the modification from the cytoplasm was not obtained (data not shown).

DISCUSSION

In the present study, we obtained the following observations:

(i) Membrane-impermeable or bulky SH-reagents abolished the efflux from cells expressing the P55C and T56C mutant, which may be due to the

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modification of the cysteine. Actually, we could show that DTNB can access the Cys56 of T56C. Note that Pro55 and Thr56 are considered to be in the cytoplasm according to the parallel topology model.

(ii) One of Thr108 residues (T108C) of the dimer faces the cytoplasm while the other Thr108 of the dimer faces the periplasm.

(iii) Cys25 (E25C) was not labelled by BM from the periplasm, but was possible from the cytoplasm. On the other hand, however, the presence of transport substrates enabled BM to modify Cys25 from the periplasm.

The observations of (*1*) and (*2*) can be easily understood by the anti-parallel configurations of the EmrE dimer (Fig. 1). On the other hand, biochemical observations suggesting the parallel configuration have already been reported (*12–14*). Hence, further study is needed to construct the structure that satisfies all the reported data.

If we assume that the flip-flop of EmrE (the rotation of Cys25 from the cytoplasm to the periplasm) does not occur by the addition of substrates, the earlier-described observation of (*3*) implies that two Cys25 (of E25C) residues in the dimer face to different sides. In addition, this indicates that the conformations of the two monomers might not be symmetric, at least when the transporter is at rest, and that the transport substrate may induce a conformational change in one monomer whose Glu25 faces the periplasm. Therefore, it might be postulated that the loop connecting TM1 and TM2 (loop 1–2, Fig. 1) might participate in the substrate-release process from EmrE. Further study of the efflux mechanism is necessary, and to this end, the precise structure in the membrane and the membrane topology are required.

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