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**Anti-parallel membrane topology of two components of EbrAB,
a multidrug transporter †**

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Abstract

EbrAB is a multidrug-resistance transporter in *Bacillus subtilis* that belongs to the small multidrug resistance, and requires two polypeptides of both EbrA and EbrB, implying that it functions in the hetero-dimeric state. In this study, we investigated the transmembrane topologies of EbrA and EbrB. Various single-cysteine mutants were expressed in *E. coli* cells, and the efflux activity was measured. Only mutants having a high activity were used for the topology experiments. The reactivity of a membrane impermeable NEM-fluorescein against the single cysteine of these fully functional mutants was examined when this reactive fluorophore was applied either from the outside or both sides of the cell membrane or in the denatured state. The results clearly showed that EbrA and EbrB have the opposite orientation within the membrane or an anti-parallel configuration.

Keywords: Efflux transporter; EmrE; Small multidrug resistance (SMR); Dual topology configuration; Fluorescein-5-maleimide (NEM-fluorescein); Ethidium efflux

Introduction

Small multidrug resistance (SMR) proteins are the smallest antiporters which expel various toxicants from bacterial cells to confer the multidrug resistance to the cell [1-3]. Interestingly, SMR is composed of two or more polypeptides whose lengths are only 100 - 120 amino acids long, and each polypeptide then forms four α -helical transmembrane helices. This implies an oligomer structure in the active form. EmrE found in *Escherichia coli* is a family archetype and is well documented to function as a homo-dimer [3-6]. EbrA/EbrB and YkkC/YkkD from *B. subtilis* and Ydge/YdgF from *E. coli* are composed of paired components that are encoded in distinct operons, and the simultaneous expression of both components is absolutely indispensable for the efflux activity [7-11]. Thus, it is most likely that the SMR transporter is generally composed of two components irrespective of the homo- or hetero-dimeric structures.

The next concern is the topology of the components within the membrane. Biochemical approaches such as chemical modification [12] and cross-linking [13,14] suggested the parallel topology of

the dimer of EmrE. A parallel topology was also reported for SugE, another SMR homologue in *E. coli* [15]. On the other hand, electron microscope (EM) data and a modeling analysis based on its EM data [4,16,17] have proposed an anti-parallel topology, which is sometimes called a "dual topology" configuration.

The anti-parallel configuration is consistent with the topology analyses of *E. coli* inner-membrane proteins performed by von Heijine and colleagues [18,19]. C-terminal tagging with alkaline phosphatase or green fluorescence protein led to the suggestion of the anti-parallel configuration of EmrE and SugE. The same analysis also predicted the anti-parallel orientation of YdgE/YdgF, one of the hetero-dimeric SMR's [18]. However, these analyses focused only on the location of the C-termini of the components. In addition, it should be noted that, in these experiments, fusion tags were attached that are much larger than SMR transporter itself; the large fusion tags might disturb the valid topology and result in a topology different from the natural one. Therefore, we may say that the essential structure of the SMR's is now under debate.

In the present study, we investigated the topology of the hetero-dimeric SMR, EbrA/EbrB. To avoid the possibility of disturbing the topology, large reporter tags are not attached. Instead, a relatively small, membrane-impermeable and SH-reactive fluorescein-5-maleimide (NEM-fluorescein)[20] is labeled to a single cysteine residue at various positions. This is one kind of "cysteine scanning" method [21]. In addition, we examined the topology of only the mutants having the normal efflux activity, which assures the valid structure of the examined protein. The obtained results strongly imply the anti-parallel topology.

Materials and Methods

Mutagenesis

To obtain a single cysteine mutant protein, a cysteine-less (CL) background mutant was first obtained by replacing the 13th position of EbrA and the 116th position of EbrB with valine and serine residues, respectively. From the CL-background, various single-cysteine 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). A single cysteine residue on the CL-background was introduced at the N-terminus, C-terminus positions and in the loop region connecting successive membrane helices as shown in Fig. 1. The loop regions were predicted by a hydropathy analysis [10] and the consideration for the amphiphilicity index of polar residues [22]. Here, the transmembrane regions are assigned so as to end with amphiphilic residues of lysine, histidine, glutamic acid, tyrosine or tryptophan.

Construction of the expression plasmid has been previously described [10]. For the simultaneous expression of EbrA and EbrB, the plasmid was obtained by inserting the gene pair of *ebrA* and *ebrB* downstream of the *tac* promoter of pFLAG-CTC (Sigma, St. Louis, MO).

Ethidium efflux assay from E. coli cells

We wanted to perform a topology analysis only for the mutant proteins whose efflux activity is normal. To estimate the activity, the efflux of ethidium, one of typical substrates of SMR, was measured, using a previously described method [10].

Topology studies using NEM-fluorescein labeling

A concentrated solution of NEM-fluorescein (20 mM; Molecular Probes, Eugene, OR) was freshly prepared in N,N'-dimethylformamide. The cells were washed twice with a buffer solution containing 400 mM NaCl and 50 mM sodium phosphate (pH 7.0), and were re-suspended at 0.5 of OD₆₆₀. This cell suspension was divided into three aliquots and saved in three test tubes (1 ml each), followed by the labeling

with the membrane-impermeable NEM-fluorescein [20] under three different conditions. They are:

(1) *Labeling from outside of the cell membrane* - NEM-fluorescein was added to the intact cell suspension at a final concentration of 0.5 mM and incubated for 30 min at room temperature while shaking. To stop the reaction and to prevent redundant labeling, β -mercaptoethanol (final concentration of 5%) was added, and the cells were washed three times with the above-described buffer supplemented with 5% β -mercaptoethanol, followed by ultrasonic disruption. The membrane fractions were collected by ultra-centrifugation at 106,000 x g for 1 h at 4 °C and solubilized in an SDS-PAGE-loading buffer containing 5% β -mercaptoethanol.

(2) *Labeling from both sides of the cell membrane* - The intact cells were disrupted by sonication in the presence of 0.5 mM NEM-fluorescein, and this suspension was allowed to be shaken for 30 min at room temperature. To stop the reaction, β -mercaptoethanol was added to a final concentration of 5% v/v. The collection and solubilization of the membrane fractions were performed in the same manner as described above.

(3) *Labeling under the denatured condition* - The intact cells were disrupted by sonication, and the membrane fractions were collected by ultra-centrifugation. The membrane fractions were then solubilized in SDS-PAGE-loading buffer containing 0.5 mM NEM-fluorescein, followed by a 30-min incubation at room temperature. Finally, β -mercaptoethanol was added at a final concentration of 5% v/v.

The final volumes of these three samples were the same, giving approximately equal protein concentrations in each container. The samples were analyzed by tricine-SDS-PAGE together with fluorescein-labeled protein standards. Fluorescence images of the gels were acquired using a Fujifilm FLA-2000 imaging system (Tokyo, Japan).

Results and Discussion

A single cysteine residue was introduced into the CL-background mutants (C13V of EbrA and C116S of EbrB) at the respective five regions of EbrA and EbrB: these regions were the N-terminus (1.5C for EbrA; G3C for EbrB), the first loop connecting the first and second membrane helices (S25C for EbrA; T28C, for EbrB), the second loop (S58C for EbrA and EbrB), the third loop (N85C for EbrA; T82C for EbrB) and the C-terminus (106C for EbrA; A106C for EbrB). Here, 1.5C and 106C for EbrA represent the mutants having cysteine residues inserted after the 1st methionine and the C-terminal proline at the 105th position, respectively. Figure 1 illustrates the possible membrane helix and loop regions together with the positions where the cysteine residues are introduced. Note that the membrane topologies of EbrA and B are drawn based on results of the present study.

The normal transport activities of these single-cysteine mutants were confirmed by the ethidium efflux assays. Figure 2 shows the time-dependent decrease in the ethidium fluorescence that represents the ethidium efflux, because the fluorescence originates

only from ethidium inside the cells [10]. Panel A is for cells expressing combinations of various single-cysteine EbrA mutants and the CL EbrB, and panel B is for cells expressing combinations of the CL EbrA and various single-cysteine EbrB mutants. All mutants maintained activities as high as that of the wild-type EbrAB (thick solid lines). The CL-background EbrAB also showed a high activity (data not shown).

The EbrAB mutants were labeled with an SH-reactive and membrane-impermeable NEM-fluorescein by three different procedures. They were labeled from the outside, from both sides and under the denatured condition, which are hereafter called "out", "both" and "all", respectively. The obtained fluorescence images are shown in Fig. 3, where "None" means the image from cells harboring a vacant plasmid. The "out" image of the "None" sample does not show clear bands, while the "all" image of the "None" shows several bands, indicating that intrinsic proteins are not labeled with the NEM-fluorescein from the outside of the intact cells and also that NEM-fluorescein does not penetrate into the inside of the cells. The image of "all" of the CL sample is almost the same

as that of "all" of the "None" sample, which is reasonable because "CL" has no cysteine residues. The presence of these intrinsic proteins fortunately did not interfere with the further analyses, because the two bands from the transporter components are much more dense than those of the intrinsic proteins. The molecular masses calculated from the amino acid compositions are 11.4 kDa for EbrA (105 A.A. residues) and 12.3 kDa for EbrB (117 A.A. residues). Thus, the upper and lower bands are from EbrB and EbrA, respectively.

We considered the results of five mutants in panel A, in which clear bands appear in all the "all" images with almost the same density. Note that the lanes are arranged in the order from the N- to C-terminus. When we focus on the "out" images, the bands alternatively appear from 1.5C to 106C. No bands appear in the "out" images of S25C and N85C. Therefore, we may conclude that the N-terminus (1.5C), Ser-58, and the C-terminus (106C) are located in the periplasmic space, while the membrane-impermeable NEM-fluorescein cannot access Ser-25 and Asn-85. Combining the hydropathy analysis, we may conclude the location of these residues as shown in the left panel of Fig. 1.

The mutant of 1.5C was constructed by the cysteine *insertion*, and hence, we examined the *replaced* mutants in the N-terminus region of EbrA. The mutants of L2C, I3C, G4C and Y5C were then constructed. The ethidium efflux activities of the G4C and Y5C mutants were weak. The topology experiments were then not done. These residues may locate around the membrane-water interface, and the cysteine replacement might disturb the protein conformation. On the other hand, the mutants of L2C and I3C showed high efflux activities, but no clear bands appeared in both the "out" and "all" images. We may consider that the N-terminus region of these mutants may be excised by unknown reasons, although we do not know the exact reasons at present.

Panel B shows the results of the combination of CL EbrA and various EbrB single cysteine mutants. Here, we can also observe the alternative appearance of a clear band in the "out" images. There is, however, an obvious difference between panels A and B, which is concerned with the "phase": +--+ in panel A while -+--+ in panel B. We may conclude that Thr-28 and Thr-82 are in the periplasmic space while Gly-3, Ser-58 and Ala-106 are located in

the cytoplasm (the right panel of Fig. 1). It is worthwhile to note that the topologies are opposite between EbrA and B (see Fig. 1). Thus, our present results reveal the anti-parallel configuration of EbrA/B. Figure 1 also indicates the location of the Lys- and Arg-residues from which we can see that the "positive-inside" rule holds.

Strictly speaking, the absence of the dense bands in the "out" images does not necessarily mean that the locations of the respective residues are in the cytoplasmic space. There is the possibility that these residues may be located within the membrane helices, although the hydropathy analysis predicts that these residues belong to the loop region connecting the two membrane helices. Therefore, we compared the band densities between the "both" and "all" images of S25C and N85C of EbrA, and G3C, S58C and A106C of EbrB (panel C in Fig. 3). The results reveal the almost equal densities of the same mutants, implying that the hydrophilic fluorescent probe can react with the cysteine residues of these mutants from the cytoplasm. In other words, the locations of these residues are in the cytoplasm as shown in Fig. 1.

In panels B and C of Fig. 3, the band positions of the EbrB mutants slightly vary. The reason for this is not clear at present. It seems that even in the presence of SDS, the 3D structure may not be completely destroyed, but it is noted that the band positions between the "out", "all" and "both" are the same within one mutant. These differences in the migration rate then do not interfere these concluding the anti-parallel configuration of EbrA/B.

Our conclusion is also consistent with previous studies on the other hetero-dimeric SMR's of YdgE/F, in which the C-terminus locations were examined using the fusion of the reporter proteins [18,19]. YdgE is a shorter member (corresponding to EbrA in this sense) whose C-terminus is located outside, and the C-terminus of the longer member, YdgF (corresponding to EbrB), is located inside. This configuration is similar with EbrA/B as shown in Fig. 1.

The conclusion of anti-parallel configuration of EbrA/B may be expanded to the membrane topology of EmrE, a homo-dimeric SMR. Contrary to EbrA/B, EmrE obviously does not have the "positive-inside" bias [19], and then we lose one of the criteria of the topology. Therefore, we will consider this from the other

aspects; i.e., What happens when the "positive-inside" bias is removed from EbrA/B? Actually this was previously done [10]. We prepared mutants of EbrA and EbrB lacking hydrophilic regions by the replacement of the positively charged lysine residues with neutral ones, and then the resultant mutants lost the clear "positive-inside" biases. Interestingly, expressing only one monomer (either of the EbrA or EbrB mutants) conferred resistance to the cell. In other words, we succeeded in preparing the homo-dimer SMR of the Ebr homologues. Although there is no direct evidence that these two monomers have the anti-parallel configuration as in the wild-EbrA/B (Fig. 1), there may be a high possibility of the anti-parallel configuration because the locations of the hydrophilic amino-acid residues participating in the substrate transport within the membrane may not be changed between the wild and the functional homo-dimer mutant. Therefore, we may infer that EmrE also has an anti-parallel configuration. This is now in progress in our laboratory.

Further investigation on the configuration of EbrA/B is needed to reveal more direct and definite evidence on the topology

shown in Fig. 1, which may be achieved by EM or X-ray analysis of the 3D crystal. Although the definite conclusion is not presently available, we may consider that SMR has the anti-parallel configuration irrespective of the home- or hetero-dimers. If this is the case, the role of the anti-parallel configuration in the membrane transport mechanism should be considered, and the functionally essential amino acid residues and their roles should be clarified based on this configuration.

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Figure Legends

Figure 1. Positions where cysteine residues were introduced on the CL-background mutants (C13V of EbrA and C116S of EbrB). The regions of the membrane α -helices (enclosed by gray boxes) and loops outside of membrane phospholipid core were predicted by the hydropathy analysis and the consideration for the amphiphilicities of polar residues. The orientations of EbrA and EbrB are drawn to be opposite, and this is a conclusion of the present paper. The positively charged residues, lysine and arginine, are highlighted by circles filled in black. The membrane topology in this figure obeys the "positive-inside" rule.

Figure 2. The confirmation that single-cysteine mutants have as high efflux activity as the wild-type. The ethidium concentrations remaining inside of the *E. coli* cells were monitored continuously by measuring the fluorescence from ethidium. The excitation and emission wavelengths were 545 nm and 610 nm, respectively. At time 0, glucose was added to energize the cells to start the ethidium efflux. The thick solid lines are the fluorescence changes from

the cells expressing the wild-type EbrA/B, and the line labeled "None" means the cells harboring a vacant plasmid. Panels (A) and (B) show the changes from the cells expressing combinations of various single-cysteine EbrA mutants and the CL EbrB mutant, and combinations of the CL EbrA mutant and various single-cysteine EbrB mutants, respectively. The positions replaced by cysteine are denoted in the figure, where the meaning of 1.5C and 106C for the single-cysteine EbrA mutants (panel A) are referenced to the text.

Figure 3. Fluorescence images of tricine-SDS-PAGE gels. Three types of labeling were done and denoted as follows: "out", labeling from the outside of the cells; "all", labeling under the denatured condition; "both", labeling from both sides of the cell membrane. Panel A shows the "out" and "all" images from the combinations of various single-cysteine EbrA mutants and CL EbrB mutant, and Panel B shows the images from the combinations of CL EbrA mutant and various single-cysteine EbrB mutants. In panels A and B, there are mutants in which NEM-fluorescein cannot react from the outside.

In panel C, the "both" images of these mutants are shown together with the "out" and "all" images.





