

Topology Determination of Untagged Membrane Proteins

Iris Nasie, Sonia Steiner-Mordoch, and Shimon Schuldiner

Abstract

The topology of integral membrane proteins with a weak topological tendency can be influenced when fused to tags, such as these used for topological determination or protein purification. Here, we describe a technique for topology determination of an untagged membrane protein. This technique, optimized for bacterial cells, allows the visualization of the protein in the native environment and incorporates the substituted-cysteine accessibility method.

Key words Topology, Untagged, Membrane protein, Cysteine, EmrE, Transport, SCAM

1 Introduction

The atomic resolution structural information on integral membrane proteins is limited, as they are more difficult to express and crystallize than water-soluble proteins [1, 2]. This has necessitated establishing other approaches to define the topology of these proteins, including the genetic fusion of tags [3–8] or reporters [9–12], and the substituted-cysteine accessibility method (SCAM) [13–16].

SCAM has been widely used to determine integral membrane protein topologies. In this technique, one noncritical residue that is thought to be in an intracellular or extracellular loop is mutated by site-directed mutagenesis to a cysteine. Cysteine residues contain sulfhydryl groups that react with a variety of sulfhydryl-specific reagents. By using neutral (membrane permeable) or charged (membrane impermeable) sulfhydryl reagents, SCAM can precisely locate the position of the introduced cysteine residue depending on its orientation—inside or outside the cell. SCAM requires a functional cysteine-less variant where a single cysteine is introduced at a designated location [17].

Genetic fusion of tags is not only used for determining the topology of integral membrane proteins, but also for the facilitation of their purification. Since tags can influence the topology of proteins with a weak topological tendency [18], the topology of these

proteins should be determined in their untagged form, which makes their purification more challenging. The method presented here is based on SCAM and obviates the need for purification of the protein. It was optimized for bacterial cells and used for the topology determination of EmrE, a transporter residing the inner membrane of *Escherichia coli* (*E. coli*) [18]. For this method, we designed single-cysteine mutations positioned in loops that are expected to be on opposite sides of the membrane. Those single-cysteine mutants were expressed in cells and specifically labeled metabolically with [³⁵S]Methionine as described in [19]. The cells were then challenged with a sulfhydryl reagent that is impermeable to the cell membrane (sodium (2-sulfonatoethyl) methanethiosulfonate; MTSES) and can therefore only react with extracellular thiol groups. After the solubilization and denaturation of the protein, the proportion of unreacted thiols was assessed with methyl-polyethylene glycol-maleimide 5,000 (Mal-PEG) [20]. Reaction with Mal-PEG results in a mass addition of 5,000 Da to the protein and therefore in a significant shift of its mobility that can be easily detected by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The results from the gel were then digitally analyzed: the band intensities were quantified and the ratio of Mal-PEG-reacted thiols to total thiols was calculated (*see* flow chart in Fig. 1a). For comparison, same procedure was done with the tagged form of these same single-cysteine mutants.

2 Materials

2.1 Specific Labeling with [³⁵S]Methionine

1. Desirable gene harboring single-cysteine mutation (in a cysteine-less background) inserted into an expression plasmid containing a phage T7 RNA polymerase promoter (*see* Note 1).
2. An *E. coli* strain transformed with the pGP1-2 “induction” plasmid. pGP1-2 encodes for the T7 polymerase under the inducible control of the λ PL promoter and contains the genes for the heat-sensitive λ repressor cI857 and kanamycin resistance (*see* Note 2).
3. 2.5 mg/ml Thiamine stock solution in double distilled water (DDW). Filter and store at room temperature (*see* Note 3).
4. 70 % (w/v) Glycerol stock solution in DDW. Autoclave and store at room temperature.
5. 10 % (w/v) MgSO₄ stock solution in DDW. Autoclave and store at room temperature.
6. 25 mg/ml Kanamycin stock solution in DDW. Filter. Leave one aliquot at 4 °C for current use and store remaining aliquots at -20 °C.

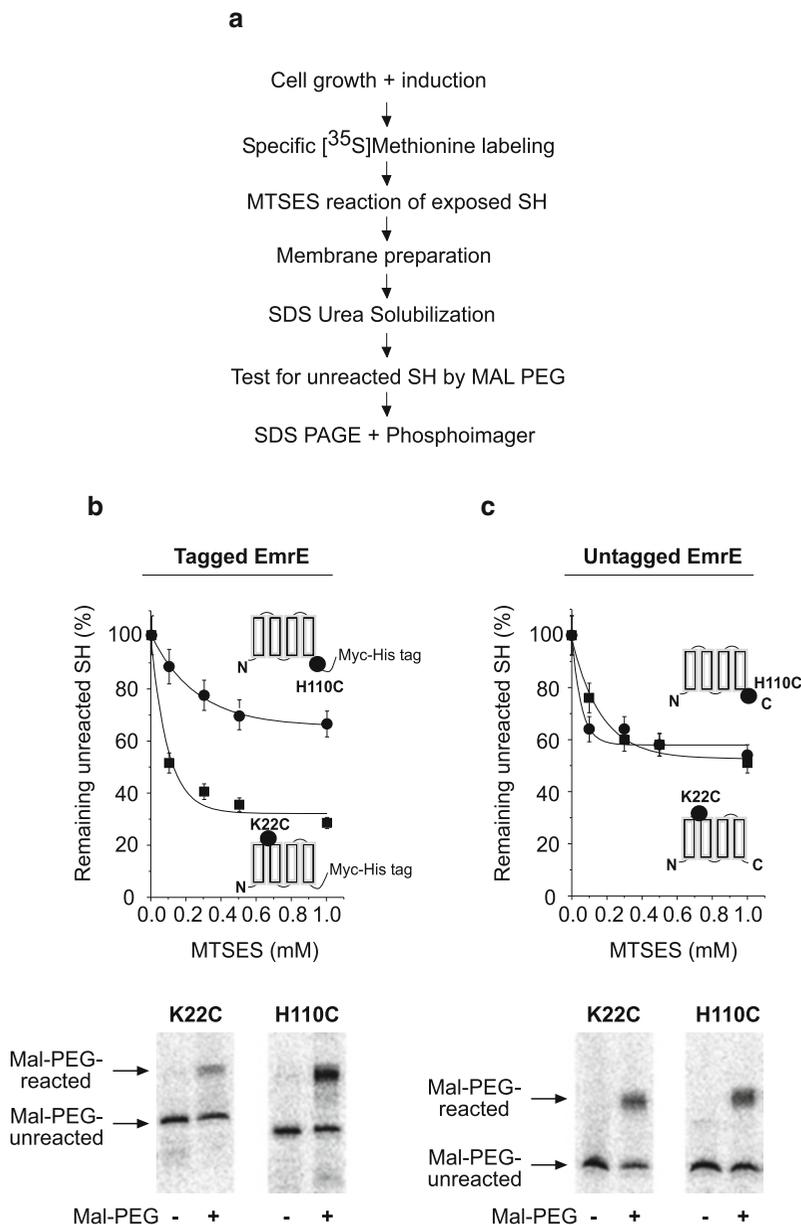


Fig. 1 Tags introduce bias in the topology of EmrE. **(a)** Simplified flow chart of procedure. Tagged **(b)** and untagged **(c)** EmrE-K22C and EmrE-H110C were metabolically labeled with [35 S]methionine in *E. coli* cells and treated with MTSES at the indicated concentrations. The unreacted thiols were estimated from the degree of reaction with Mal-PEG. EmrE that reacted with Mal-PEG displays a higher apparent molecular weight (M_r) in SDS-PAGE (**(b, c)** bottom panels, compare lanes with and without Mal-PEG). The ratios of MTSES-unreacted thiols (intensity of higher M_r bands) over total thiols (intensity of higher M_r bands + intensity of lower M_r bands) were calculated using Image Gauge 3.46 Fujifilm software and are shown in the graphs. The bottom panels in **(b)** and **(c)** are samples of the types of changes at one concentration of MTSES: 0.3 mM and 0.1 mM, respectively. Figure originally published in The Journal of Biological Chemistry © Nasie, I., Steiner-Mordoch, S., Gold, A. and Schuldiner S. (2010) Topologically random insertion of EmrE supports a pathway for evolution of inverted repeats in ion-coupled transporters 285, 15234–44. The American Society for Biochemistry and Molecular Biology

7. Minimal Medium A (MMA) + supplements.

Minimal Medium A (×1)—60 mM K_2HPO_4 , 33 mM KH_2PO_4 , 7.5 mM $(NH_4)_2SO_4$ (*see Note 4*).

Supplements (final concentrations)—2.5 µg/ml thiamine, 0.01 % $MgSO_4$, 0.5 % glycerol (*see Note 5*), 50 µg/ml kanamycin, and a suitable antibiotic for the selection of expression plasmid (we used 100 µg/ml ampicillin for pT7-7).

8. 20 mg/ml Rifampicin stock solution in methanol (*see Note 6*).
9. L-[^{35}S]methionine (specific activity, >1,000 Ci/mmol) (Institute of Isotopes Co., Ltd.). Store at 4 °C (*see Note 7*).
10. 2 M NaCl stock solution in DDW. Autoclave and store at room temperature.
11. 1 M Tris-HCl pH 7.5 stock solution. For 500 ml: weigh 60.5 g Tris-Base and transfer to a 500 ml measuring cylinder. Add DDW to a volume of 400 ml. Mix on a magnetic stirrer up to dissolving and adjust pH with HCl (*see Note 8*). Make up to 500 ml with DDW. Autoclave and store at room temperature.
12. Na buffer: 150 mM NaCl, 15 mM Tris-HCl pH 7.5. Store at room temperature.

2.2 Topology Determination

1. Na buffer: *see* Subheading 2.1 [12].
2. 1 M $MgSO_4$ stock solution in DDW. Autoclave and store at room temperature.
3. 50 mM MTSES (sodium (2-sulfonatoethyl) methanethiosulfonate) (Anatrace, Inc.) stock solution in DDW (*see Note 9*).
4. 50 % (w/v) Sucrose stock solution in DDW. Autoclave and store at room temperature.
5. 1 M Tris-HCl pH 8.0 stock solution in DDW. Preparation procedure as in Subheading 2.1 [11].
6. 5 mg/ml Lysozyme stock solution in DDW. Prepare fresh.
7. 0.5 M EDTA stock solution in DDW. Autoclave and store at room temperature.
8. 1.5 µg/ml DNaseI stock solution in 5 mM NaAc, 1 mM $CaCl_2$ pH 4.6–5.0 (titrated by acetic acid). Leave one aliquot at 4 °C for current use and store remaining aliquots at –20 °C.
9. Solubilization solution: 2 % (w/v) SDS, 6 M Urea, 15 mM Tris-HCl, pH 7.5 (*see Note 10*).
10. 50 mM Mal-PEG 5,000 (methyl-polyethylene glycol-maleimide; M_R 5,000) (Nektar Transforming Therapeutics, Huntsville, AL) stock solution in DDW. Prepare fresh. Store at 4 °C.

2.3 SDS-Urea Polyacrylamide Gel Electrophoresis

1. Gel buffer: 3 M Tris-HCl, pH 8.45, 0.3 % (w/v) SDS. For 200 ml: Weigh 72.6 g Tris-Base and transfer to a 250 ml measuring cylinder. Add water to a volume of 100 ml. Mix on a magnetic stirrer up to dissolving and adjust pH with HCl (*see Note 8*). Make up to 200 ml with water. Add 600 mg SDS and mix gently (*see Note 11*). Store at room temperature.
2. 40 % Acrylamide/Bis solution (29:1 acrylamide:Bis). Store at 4 °C (*see Note 12*).
3. 10 % (w/v) Ammonium persulfate stock solution in water. Store at 4 °C (*see Note 13*).
4. *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED). Store at 4 °C (*see Note 14*).
5. Separating gel: gel buffer (×1) (1 M Tris-HCl, pH 8.45, 0.1 % (w/v) SDS), 16 % acrylamide/Bis solution (29:1 acrylamide:Bis), 10 % (w/v) Glycerol, 6 M Urea.
6. Stacking gel: gel buffer (×1) (1 M Tris-HCl, pH 8.45, 0.1 % (w/v) SDS), 4% acrylamide/Bis solution (29:1 acrylamide:Bis), 6 M Urea.
7. Cathode buffer (×10): 1 M Tris-Base, 1 M Tricine, pH 8.25, 1 % (w/v) SDS. For 500 ml: Weigh 60.5 g Tris and 89.6 g Tricine and transfer to a 500 ml measuring cylinder. Add water to a volume of 400 ml. Mix on a magnetic stirrer up to dissolving and adjust pH with Tricine (*see Note 8*). Make up to 500 ml with water. Add 5 g SDS and mix gently (*see Note 11*). Store at room temperature (*see Note 15*).
8. Anode buffer (×10): 2 M Tris-HCl, pH 8.9. For 500 ml: Weigh 121 g Tris-Base and transfer to a 500 ml measuring cylinder. Add water to a volume of 400 ml. Mix on a magnetic stirrer up to dissolving and adjust pH with HCl (*see Note 8*). Make up to 500 ml with water. Store at room temperature (*see Note 15*).
9. Sample buffer (×6): 0.3 M Tris-HCl pH 6.8, 12 % (w/v) SDS, 0.6 % (w/v) bromophenol blue, 0.6 M β-mercaptoethanol, 60 % (v/v) Glycerol. Leave one aliquot at 4 °C for current use and store remaining aliquots at -20 °C (*see Note 16*).

3 Methods

3.1 Heat-Induced Over-expression and Specific Labeling with [³⁵S]Methionine

Day 1

Transform expression plasmid containing desirable gene into the pGPI-2 containing strain (*see Note 17*). Incubate at 30 °C (*see Note 18*).

Day 2

Grow cells at 30 °C in minimal medium A+supplements, over night with shaking (200 rpm) (*see Note 19*).

Day 3

1. Dilute cells to $A_{600}=0.1$ into 50 ml of fresh MMA+supplements (use an Erlenmeyer of 250 ml). Grow at 30 °C.
2. When the culture reaches an $A_{600}=0.6$, transfer it to a water bath at 42 °C with shaking to induce the T7 polymerase expression. Incubate for 15 min.
3. Add rifampicin (final CONC. 200 µg/ml), and continue incubating with shaking for an additional 10 min at 42 °C (*see Note 20*).
4. Shift the culture back to 30 °C for 40 min.
5. Add L-[³⁵S]methionine (final CONC. 10 µCi/ml), and continue incubating at 30 °C for an additional 35 min (*see Note 21*).
6. Collect cells by centrifugation (4 °C, 3,200×g, 10 min). Discard supernatant. Put on ice.
7. Wash cells by an addition of 50 ml of ice-cold Na buffer followed by centrifugation (4 °C, 3,200×g, 10 min). Discard supernatant. Put on ice. Repeat washing once more with 10 ml of same solution (*see Note 22*).

**3.2 Topology
Determination
(and SDS-Urea PAGE)**

Carry out all procedures at room temperature unless otherwise specified.

1. Resuspend pellet with 5.5 ml of Na buffer containing 5 mM MgSO₄ (*see Note 23*).
2. Divide cells into aliquots of 1 ml (~10 ml of cultured cells per assay), harvest them by centrifugation (4 °C, 21,000×g, 1 min), and resuspend pellet with 200 µl of Na buffer containing 5 mM MgSO₄.
3. Add increasing concentrations of MTSES (0, 0.1, 0.3, 0.5, 1.0 mM) and incubate samples at 30 °C for 20 min.
4. Remove MTSES by addition of 1 ml Na buffer and centrifugation (4 °C, 21,000×g, 2 min).
5. Rinse cells three times with 1 ml Na buffer (4 °C, 21,000×g, 2 min).
6. Lysis: resuspend cells pellet with 150 µl of a solution containing 30 % (w/v) sucrose, 30 mM Tris-HCl, pH 8.0, 50 µg/ml lysozyme, and 10 mM EDTA. Incubate at 37 °C for 15 min.
7. Proceed lysis by the addition of 900 µl of DDW containing 0.15 µg/ml DNaseI and 15 mM MgSO₄. Incubate at 37 °C for 15 min.

8. Collect membrane fraction by centrifugation (4 °C, 21,000 ×g, 20 min). Resuspend membranes in 50 µl Na buffer.
9. Measure radioactivity: take 2 µl of membrane sample into a scintillation tube and add 18 µl of DDW. Add scintillation liquid, mix, and read cpm in a beta-counter.
10. For each sample, take a membrane volume corresponding to ~200,000 cpm. For membrane solubilization add 20 µl of solution containing 2 % (w/v) SDS, 6 M urea, 15 mM Tris-HCl, pH 7.5, in the presence of 2.5 mM Mal-PEG 5,000, and incubate with shaking at 30 °C for 1 h.
11. Stop the reaction by the addition of 4 µl sample buffer (×6).
12. Load protein samples on 16 % Tricine SDS-Urea PAGE.
13. Visualize the radioactive bands with a Phosphor-Imager (*see Note 24*).
14. Use suitable software to digitally analyze the intensity of the radioactive bands (*see Note 25*).
15. Calculate the ratios of MTSES-unreacted/unexposed thiols (intensity of higher molecular weight bands) over total thiols (intensity of higher molecular weight bands+intensity of lower molecular weight bands).

3.3 An Example for Results Obtained for EmrE Using the Technique

EmrE is a transporter residing in the inner membrane of *E. coli* and composed of four transmembrane helices connected by short hydrophilic loops. EmrE has only a weak topological tendency, meaning that it has no specific tendency to assume a topology of N_{in}-C_{in} (both termini are toward the cytoplasm) or N_{out}-C_{out} (both termini are toward the periplasm). We studied its topology using the SCAM technique. For that, we used cysteine (Cys)-less untagged EmrE, and also Cys-less tagged EmrE, as a control (the protein is fused at its C terminus to a Myc epitope followed by a hexa-histidine tag). In each construct, we engineered the single Cys residue to be at position 22 (substitution of lysine 22; loop 1) or at position 110 (substitution of histidine 110; C terminus); these two residues are expected to be on either side of the membrane according to hydrophathy analysis. The mutants were expressed in cells and specifically labeled metabolically with [³⁵S] methionine. The cells were then challenged with the impermeant reagent, MTSES. After the solubilization and denaturation of the protein, the available thiols that did not react with the MTSES were assessed with Mal-PEG.

The topological results obtained for EmrE are shown in Fig. 1. In tagged mutants, cysteine in loop 1 at position 22 (EmrE-K22C) but not at the C terminus, position 110 (EmrE-H110C), reacted with MTSES in the intact cell, therefore only a fraction of ~30 % of the protein reacted with Mal-PEG (Fig. 1b). However, with untagged mutants bearing Cys at the same positions, reactivity of

both EmrE-K22C and EmrE-H110C was very similar and partial; ~50–60 % of the residues are exposed in each case (Fig. 1c). These results show that the untagged protein displays a dual topology, i.e., approximately half of the protein is $N_{in}-C_{in}$, whereas the other half is $N_{out}-C_{out}$, as expected for a protein with a weak topological tendency. In contrast, the Myc-His tag is biasing the protein towards a $N_{in}-C_{in}$ topology.

4 Notes

1. We use the pT7-7 expression plasmid which confers resistance to ampicillin [19].
2. It is also possible to induce gene expression in the BL21(DE3) strain [21], where the gene coding for the T7 polymerase has been inserted into the chromosome under the inducible control of the *lacUV5* promoter, but we found that the labeling is more efficient when the expression is heat-induced.
3. Thiamine stock solution should be protected from light, since it is light-sensitive.
4. Prepare Minimal medium A ($\times 10$). Autoclave and store at room temperature. Right before use, dilute tenfold with DDW and add the supplements.
5. 0.5 % Glucose can be used as carbon source instead of 0.5 % Glycerol. Prepare 20 % (w/v) Glucose stock solution in DDW. Autoclave and store at room temperature.
6. Methanol should be handled with appropriate precautionary measures in accordance with safety procedures. Prepare Rifampicin stock solution fresh. Protect from light since Rifampicin is light-sensitive. Rifampicin inhibits transcription of the *E. coli* RNA polymerase without affecting the phage T7 polymerase which is responsible for the transcription of the genes to be over-expressed. This allows the exclusive labeling of the protein of interest [19].
7. Should be handled with appropriate precautionary measures in accordance with local radioactive safety procedures. The radioactive labeling is for the detection of the unpurified untagged protein. Detection of the protein can also be done by using a suitable antibody instead.
8. Tris can be dissolved faster provided the water is warmed to about 37 °C. However, the downside is that care should be taken to bring the solution to room temperature before adjusting pH.
9. Prepare right before use because MTSES has a very short half life [22].

10. First dissolve the Urea with DDW, then add the Tris-HCl, and eventually add the SDS.
11. SDS has a tendency to create foam when mixed on a magnetic stirrer too vigorously.
12. Harmful substance that should be handled according to its material safety data sheet (MSDS).
13. We find that it is best to use it for no more than a week.
14. Harmful substance that should be handled according to its MSDS. We find that storing at 4 °C reduces its pungent smell.
15. Before running a gel, dilute cathode and anode buffers by 10.
16. SDS precipitates at 4 °C. Therefore, the sample buffer needs to be warmed prior to use.
17. Spread transformed bacteria on a plate containing antibiotics that select for each of the plasmids. We used plates containing ampicillin and kanamycin for the selection of pT7-7 and pGPI-2, respectively.
18. The induction is done at 42 °C which is close to 37 °C. Thus, to avoid preinduction, cells are incubated at 30 °C. Cells exposed to higher temperatures should be discarded.
19. Since growth in a minimal medium is slower, add two to three colonies to each tube that should contain no more than 2 ml, and incubate starters for a minimal time of ~20 h.
20. Add while the Erlenmeyers are still in incubator. To avoid loss of rifampicin, make sure to pipette it close to the Erlenmeyer, as methanol tends to drip.
21. Should be handled with appropriate precautionary measures in accordance with local radioactive safety procedures. Add while the Erlenmeyers are still in incubator.
22. Pellet can be stored at -70 °C. We did not.
23. If pellet was stored at -70 °C, thaw it in 37 °C water bath before resuspending.
24. We use the FLA-3000 Phosphor-Imager; Fujifilm, Tokyo.
25. We use the Image Gauge 3.46 Fujifilm software.

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