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Site-directed alkylation of cysteine to test solvent accessibility of membrane proteins

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Abstract

This protocol describes a detailed method to study the static and dynamic features of membrane proteins, as well as solvent accessibility, by utilizing the lactose permease of *Escherichia coli* (LacY) as a model. The method relies on the use of functional single-Cys mutants, an affinity tag and a PhosphoImager. The membrane-permeant, radioactive thiol reagent *N*-[ethyl-1-¹⁴C]ethylmaleimide ([¹⁴C]NEM) is used to detect site-directed alkylation of engineered single-Cys mutants *in situ*. The solvent accessibility of the Cys residues is also determined by blockage of [¹⁴C]NEM labeling with membrane-impermeant thiol reagents such as methanethiosulfonate ethylsulfonate (MTSES). The labeled proteins are purified by mini-scale affinity chromatography and analyzed by gel electrophoresis. Gels are dried and exposed to a PhosphoImager screen for 1–5 d, and incorporation of radioactivity is visualized. Initial results can be obtained in 24 h.

INTRODUCTION

Chemical modification is a simple, useful approach to study membrane protein structure and function. Among amino acids, Cys is average in steric bulk, relatively hydrophobic and amenable to highly specific modification. Cys-scanning mutagenesis takes advantage of these unique features of Cys combined with site-directed mutagenesis^{1,2}. In order to optimize the approach, it may be necessary to construct a nonreactive or Cys-less mutant without inactivating the protein. On a functional Cys-less background, by systematically mutating each residue to Cys, a library of single Cys-mutant is generated, and the functional role of each position can be assessed by testing activity. A further advantage of the approach is that it enables studies of modification by Cys-specific reagents.

Site-directed sulfhydryl modification of single-Cys mutants *in situ* with radioactive *N*-ethylmaleimide (NEM) has been particularly useful for studying both static and dynamic features of the lactose permease of *Escherichia coli* (LacY)³. In this protocol, LacY is used as a prototype^{4,5}. Alkylation with NEM is a measure of the reactivity and/or accessibility of a given Cys residue to this small, relatively hydrophobic, membrane-permeant thiol-specific reagent. Reactivity and/or accessibility are dependent primarily on the environment in the vicinity of a given Cys side chain and limited by close tertiary contacts between transmembrane

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helices and steric constraints of the lipid bilayer. Any change in reactivity of a Cys side chain upon substrate binding is indicative of an alteration in the local environment. Hence, determination of the reactivity of Cys replacement mutants with *N*-[ethyl-1-¹⁴C]ethylmaleimide ([¹⁴C]NEM) is a convenient way to assess the local environment of specific positions within the tertiary structure of the protein. Furthermore, *in situ* site-specific reaction with methanethiosulfonate ethyl-sulfonate (MTSES), a small hydrophilic, membrane-impermeant thiol reagent^{6,7}, can be utilized to study the accessibility of Cys residues to the aqueous milieu. Cys-scanning mutagenesis and site-directed sulfhydryl modification systematically applied to LacY has provided enormously valuable information with regard to structure, function and dynamics^{2,3,5,8}.

Here, we describe a simple, easy-to-handle protocol for measuring the reactivity of single-Cys mutants with various thiol reagents. Application allows (Fig. 1 and Fig. 2) (i) assessment of Cys reactivity with NEM under various conditions (e.g., absence or presence of ligand and/or an electrochemical proton gradient, temperature) (Fig. 1a and Fig. 2a) and (ii) assessment of Cys reactivity with other nonradioactive thiol reagents by measuring blockade of radioactive NEM labeling (Fig. 1b and Fig. 2b). Once Cys reacts with other thiol reagents, it cannot react with [¹⁴C]NEM. One such application includes the use of impermeant MTSES^{6,7,9} to study solvent accessibility; (iii) another application is estimation of apparent binding constants for a ligand by measuring ligand protection against alkylation with [¹⁴C]NEM^{10,11} (Fig. 1c), which will not be described here. In principle, NEM labeling and solvent accessibility approaches can be applied to identify residues buried in the core of a soluble protein by carrying out the analyses in the native or denatured condition. Furthermore, it is also useful for identifying positions located in the protein–protein interface of a protein complex by studying the effect of chemical modification on protein–protein interactions, solvent accessibility, as well as the protection against the alkylation of Cys residues.

Limitations of this particular protocol include requirement for radioactive NEM, use of an affinity tag fused to the target protein and a target protein containing only a single reactive Cys residue. Several affinity tags can be used to purify the target protein, examples being a His-tag on the target protein and metal affinity chromatography or other commercially available tags and protein purification kits. Here, we describe a protocol for purifying LacY containing a biotin acceptor domain at the C terminus by avidin chromatography^{12,13}.

It is important that the membrane permeability of the thiol reagent be tested in each system. In *E. coli*, NEM and MTSES are demonstrated to be permeant and impermeant, respectively, by analyzing labeling of cytoplasmic proteins⁹. It is also highly noteworthy that alternative methods with fluorescent thiol reactive reagents have been utilized^{14–16}.

MATERIALS

REAGENTS

- [¹⁴C]NEM, 1.3 GBq mmol⁻¹ (34.20 mCi mmol⁻¹) 0.5 ml Pentane (DuPont NEN, Boston, MA) **! CAUTION** Pentane is volatile and irritant. It is harmful by inhalation, ingestion or skin absorption. **! CAUTION** All experiments that involve the use of [¹⁴C]NEM must be carried out following radiation safety guidelines.
- MTSES (Toronto Research Chemicals, Toronto, Ontario, Canada)
- Right-side out (RSO) membrane vesicles containing the target protein with an affinity tag can be prepared as described previously^{17,18}.
- 100 mM potassium phosphate (KP_i) or sodium phosphate (NaP_i), (pH 7.5)
- 100 mM KP_i (pH 7.5)/10 mM magnesium sulfate (MgSO₄)

- 10% Dodecyl- β -D-maltopyranoside (DDM; Anatrace, cat. no. D310A) ▲ **CRITICAL** Use NaP_i because KP_i will precipitate with SDS.
- 1 M DTT (Sigma-Aldrich, cat. no. D0632)
- Immobilized monomeric avidin gel (Pierce, cat. no. 20228) ▲ **CRITICAL** Further treatment with biotin is required, as described in the product information package from the supplier, Pierce.
- Column wash buffer (see REAGENT SETUP)
- Elution buffer (see REAGENT SETUP)
- Sample loading buffer [SDS–polyacrylamide gel electrophoresis (SDS-PAGE)]

EQUIPMENT

- 15-ml conical glass tube ! **CAUTION** Do not use plastic tubes with pentane, as this solvent may dissolve certain plastics.
- 1.5-ml conical screw cap tube with O-ring (VWR Scientific Products, cat. no. 20170-110)
- Argon gas
- Gel dryer (SpeedFEL; Savant, cat. no. SG210D)
- Gel blot paper (Scheicher & Schuell, cat. no. BC 013)
- White light box
- Storage phosphor screen and exposure cassette (Molecular Dynamics)
- Kodak intensifying screen cleaner (Kodak, cat. no. 1064930)
- EL Mylar (Fralock, cat. no. F430052404, 0005 inch, 8 × 10)
- PhosphoImager
- –80 °C Freezer
- 3-ml Syringe barrels
- DNA miniprep column
- Vacuum manifold (Promega, cat. no. A7231) (Fig. 3)
- Microfuge
- Eppendorf tube
- Vertical electrophoresis apparatus

REAGENT SETUP

Column wash buffer 50 mM NaP_i (pH 7.4)/0.1 M NaCl/0.02 % DDM.

Elution buffer 5 mM Biotin in the column wash buffer given above; adjust to pH 7.5.

PROCEDURE

Preparation of [^{14}C]NEM solution ● **TIMING 1–2 h**

1. Add 0.5 ml H_2O to a 15-ml conical glass tube.

2. Tap the vial containing the [^{14}C]NEM solution on the bench top to force the reagent to the bottom of the vial.
3. Place vial containing [^{14}C]NEM on ice, in order to avoid pressure built-up, and file the top of the vial.
4. In a hood, break glass manually wearing gloves and using a kimwipe.
5. Immediately transfer the vial content to the 15-ml conical tube into the 0.5 ml H_2O (see Step 1) and mix on vortex immediately to avoid evaporation. Mark the interface of the two immiscible solvents.
6. Bubble the two-layer mixture gently with argon to evaporate the upper pentane layer (on completion, only one phase will be observed after mixing).
7. Aliquot the solution from Step 6 to a 1.5-ml conical screw cap tube with an O-ring screw top.

■ **PAUSE POINT** Store at -80°C until use. The NEM solution should be stable for at least a few months.

Preparation of membrane vesicle suspension ● **TIMING 20 min**

- 8 Place 50 μl of RSO membrane vesicles at a concentration of 20 mg protein ml^{-1} (approximately 0.1 mg of the target protein) in 100 mM KP_i (pH 7.5)/10 mM MgSO_4 in a 1.5-ml Eppendorf tube. The protein concentration can be estimated by the measurement at OD_{600} . To obtain an accurate measurement, vesicles must be diluted to a lower concentration (less than 1.0 at OD_{600}). An OD_{600} of 1 corresponds to approximately 1 mg protein ml^{-1} .

Blockade of [^{14}C]NEM labeling with MTSES ● **TIMING 1–2 h (optional)**

- 9 Add to the vesicle suspension MTSES to a final concentration of 0.2 mM, and incubate for 5 min in the absence or presence of ligand, (see Fig. 1b and Fig. 2b). Stop reaction by dilution with 1.4 ml ice-cold 100 mM KP_i (pH 7.5)/10 mM MgSO_4 and centrifuge to remove excess reagents.
- 10 Wash another two times with 1.4 ml ice-cold 100 mM KP_i (pH 7.5)/10 mM MgSO_4 to remove the remaining MTSES (Fig. 2b).
- 11 Resuspend vesicles in 50 μl of 100 mM KP_i (pH 7.5)/10 mM MgSO_4 .
- 12 For those samples to which a ligand had been added before the addition of MTSES (see Step 9), add the same concentration of ligand back to the samples.

[^{14}C]NEM labeling ● **TIMING Approximately 30 min to 2h**

- 13 To the Eppendorf tube, add 12 μl aqueous solution of [^{14}C]NEM from Step 7 to a final concentration of 0.5 mM, and start the timer.
- 14 At the appropriate time, add 1 μl of 1.0 M DTT to quench the reaction, mix on vortex and immediately place on ice. Time of labeling may vary with different membrane proteins or with different single-Cys mutants in the same protein. With LacY, labeling for 10 min may represent a rate of reaction for most positions^{11,19}. When testing solvent accessibility (see Steps 9–12), the incubation time for [^{14}C]NEM labeling should be prolonged to 30–60 min.

Purification of biotinylated protein ● **TIMING** Approximately 30 min

- 15 Following reaction quenching with DTT, add 40 μ l 100 mM KP_i or NaP_i (pH 7.5) to the suspension.
- 16 Add 25 μ l 10% DDM to a final concentration of 2%.
▲ CRITICAL STEP Type of detergent and its concentration must be tested for each individual membrane protein. To obtain this information, a wide range of detergents must be screened in order to find those that solubilize the target membrane protein and maintain its stability in solution. This can be achieved by ultracentrifugation of the sample after addition of a given detergent and carrying out a western blot on the supernatant.
- 17 Mix by flicking the tube several times. The sample should clear immediately.
- 18 Add 40 μ l immobilized monomeric avidin gel and mix by flicking the tube several times.
- 19 Incubate on a rotating platform at room temperature (~ 20 °C) for 5 min or at 4 °C for 30 min.
- 20 Place a Promega wizard column or any other DNA miniprep column on a vacuum manifold and prepare 3-ml syringe barrels (Fig. 3).
- 21 Spin down the sample, resuspend avidin gel with pipette tips and apply the sample to the column.
- 22 Turn on manifold just long enough to drain fluid.
- 23 Rinse sample tube with column wash buffer, apply to column and turn on the manifold briefly to drain the mixture.
▲ CRITICAL STEP Column wash buffer must have the detergent to avoid the aggregation of protein.
- 24 Attach syringe barrel to column.
- 25 Wash column with 6 ml column wash buffer.
- 26 Turn off manifold as soon as buffer is depleted.
- 27 Remove syringe barrel.
- 28 Place the column to a 1.5-ml Eppendorf tube.
- 29 Add 50 μ l of 5 mM biotin in column wash buffer to top of column.
- 30 Wait for 2 min.
- 31 Spin at 12,000 r.p.m. for 20 s. Discard the columns in radioactive trash bin.
- 32 Purified protein samples in the Eppendorf tube are ready for analysis.

Separation and analysis of [^{14}C]NEM-modified LacY ● **TIMING** 3–4 h

- 33 To the protein sample from Step 32 add 5 μ l 10 \times SDS-PAGE sample loading buffer. Load two aliquots of 5 or 50 μ l of the resulting solution onto two different 12% SDS-PAGE gels without heating the samples. Load protein markers to each gel.
▲ CRITICAL STEP Do not heat sample; heating causes aggregation of hydrophobic membrane proteins.

- 34 Run electrophoresis of the two gels at 20 mA and stop electrophoresis before the blue bands reach bottom (approximately 1–2 h).
- 35 Use one gel for western blot to detect the loaded protein. This works as an internal control.
- 36 Place the other gel onto gel blot paper, put onto Gel Dryer, covering gel with Saran wrap.
- 37 Dry gel at 80 °C for 1 h.
- 38 During this time, clean storage phosphor screen with Kodak intensifying screen cleaner and a soft cotton cloth. Erase the storage phosphor screen by placing facedown onto a light box until use (expose for approximately 1 h).
 - ! CAUTION Carefully treat the storage phosphor screen as glass.
- 39 Trim the gel blot paper around the gel and tape the gel down to an exposure cassette.
- 40 Cover gel with a sheet of EL Mylar.
- 41 Expose by placing the blank storage phosphor screen directly onto the exposure cassette, lock the cassette and be sure that there is no exposure of the screen to light.
- 42 Label the exposure cassette with date, and store it flatly in a bench drawer at a room temperature.
 - PAUSE POINT Exposure usually takes 1–5 d.

Scan with PhosphorImager ● TIMING 1–5 d

- 43 On the second day, scan the storage phosphor screen in PhosphoImager. Density of the band at the position corresponding to that of target protein represents [¹⁴C]NEM-labeled protein. Save image as a ‘tiff’ file to be displayed through Adobe Photoshop software. This is the day-1 result, which should provide useful information to adjust the exposure time to obtain a high quality image. Intensity of the protein band is linear over a wide dynamic range. If the signal is weak, you need a longer exposure by simply erasing the image and exposing it again as described next.
- 44 Repeat Steps 38, 41 and 42. On the fifth day or the days after it, scan the storage phosphor screen in PhosphoImager to obtain a new image. If the image is still too weak, erase it and perform the exposure for a longer time.
- 45 When finished, discard gel in radioactive trash bin.
- 46 Clean the storage phosphor screen as described in Step 38 so that it is ready for further use, and return the screen to the exposure cassette.

? TROUBLESHOOTING

● TIMING

- Cast SDS-PAGE: 2 h
- Steps 1–7, preparation of [¹⁴C]NEM solution: 1–2 h
- Step 8, preparation of membrane vesicles suspension: 20 min
- Steps 9–12, MTSES labeling: 1–2 h
- Steps 13 and 14, [¹⁴C]NEM labeling: approximately 30 min to 2h
- Steps 15–32, purification of biotinylated protein: approximately 30 min

Steps 33–42, separation and analysis of [¹⁴C]-NEM–modified LacY: 3–4 h

Steps 43–46, exposure: 1–5 d or longer

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

The reactivity of Cys residues with NEM as well as the effect of ligand can be easily visualized by comparing the density of radioactive bands (Fig. 4a, upper panel). The total protein loaded, as visualized from western blot (Fig. 4a, lower panel) and data collected from a positive and a negative control for protein labeling are needed for a correct interpretation of the results. The following are examples for the interpretation of results as reported from studies on solvent accessibility of single-Cys residues determined by blockade of NEM labeling with MTSES following the protocol described above (Fig. 4a). The position studied is mapped in an x-ray crystal structure of LacY (Fig. 4b,c).

1. *L329C LacY*²⁰. NEM labeling is nearly completely blocked by pretreatment with MTSES (compare lanes 1 and 3), showing that this Cys residue is highly accessible to solvent. The presence of the ligand β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) at a saturating concentration does not alter accessibility (compare lanes 1 and 3 to lanes 2 and 4). Consistently, Leu³²⁹ (helix X) is exposed to the hydrophilic cavity of LacY (Fig. 4b,c).
2. *Q60C LacY*²¹. NEM labeling is blocked by MTSES (compare lane 5 to lane 7), showing that Cys at position 60 is highly accessible to solvent. In the presence of ligand, the side chain becomes less accessible to solvent, as shown by the decreased effectiveness of MTSES as a blocking agent (compare lanes 7 and 8). Gln⁶⁰ (helix II) is fully exposed to the hydrophilic cavity (Fig. 4b,c).

Notably, Cys at positions 329 (native residue Leu) and 60 (native residue Gln) both are accessible to MTSES. Since these positions are located on the cytoplasmic side of transmembrane helices and MTSES is membrane impermeant, the reactivity observed may be due to conformational dynamics of LacY (i.e., exposure of positions lining the hydrophilic cavity).

3. *V315C LacY*²⁰. Although the presence of ligand markedly increases NEM labeling (compare lanes 9 and 10), a Cys side chain at position 315 is not accessible to MTSES (compare lanes 10 and 12), indicating lack of exposure to bulk solvent. Val³¹⁵ (helix X) is located between helical X and VII.
4. *T45C LacY*²¹. In the presence of ligand, an increase in NEM labeling is observed (compare lanes 13 and 14), and the side chain is accessible to MTSES (compare lanes 14 and 16). Thr⁴⁵ (helix II) is located between helical II, I and VII.

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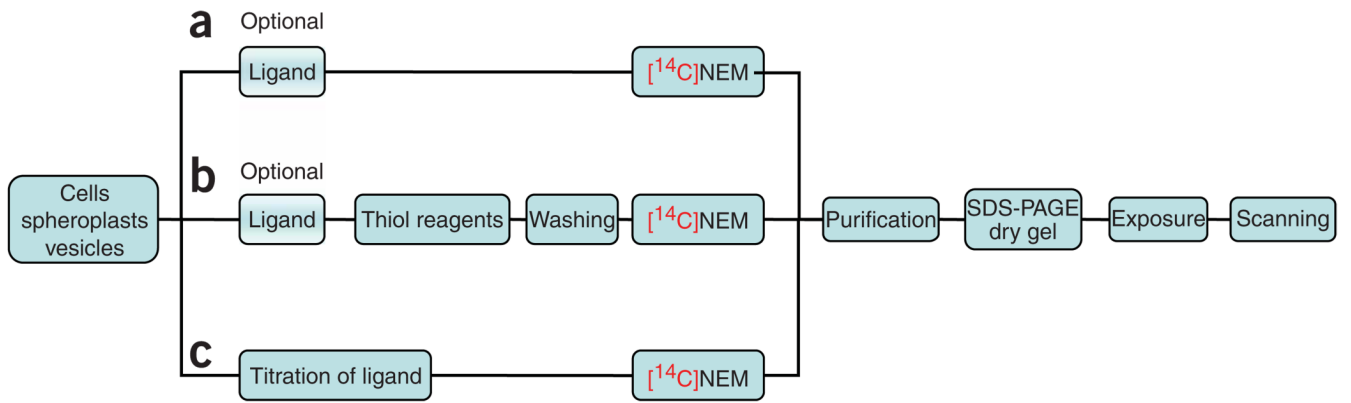


Figure 1.

Diagram for the application of *N*-ethylmaleimide (NEM) labeling. [¹⁴C]NEM, *N*-[ethyl-1-¹⁴C]ethylmaleimide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

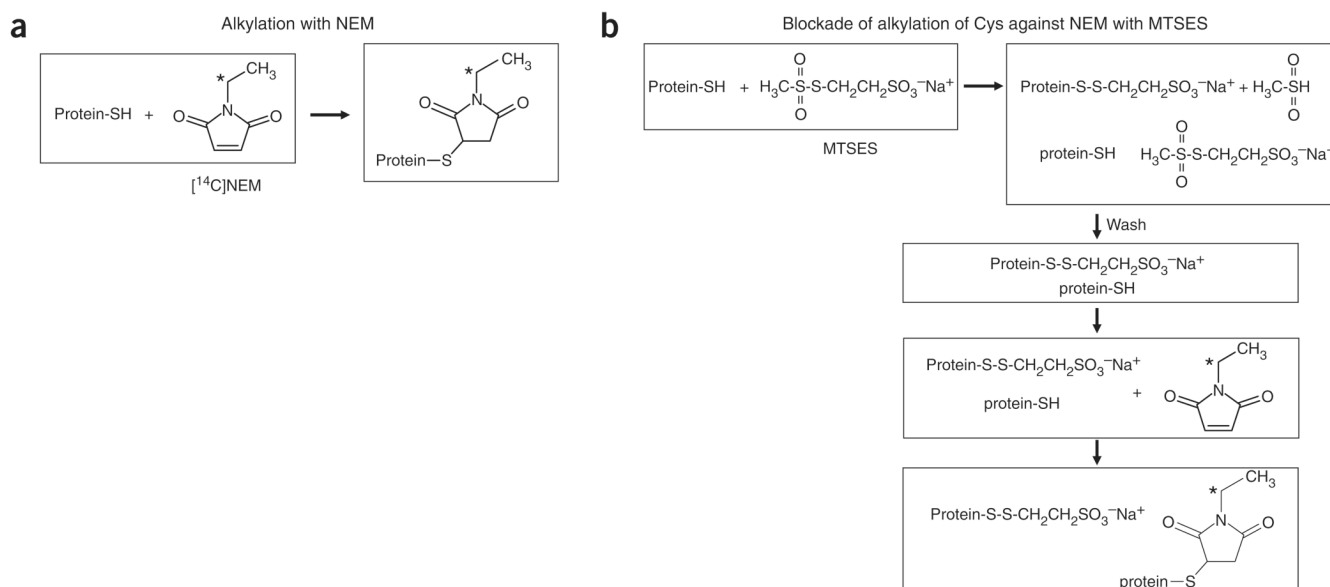
**Figure 2.**

Diagram for the strategy of testing solvent accessibility of Cys. **(a)** Chemical reaction of the membrane permeant *N*-ethylmaleimide (NEM) with Cys. **(b)** Detection of the reaction of the membrane-impermeant methanethiosulfonate ethylsulfonate (MTSES). When Cys residue partly reacts with MTSES, the remaining Cys will be detected by *N*-[ethyl-1-¹⁴C] ethylmaleimide ($[\text{14C}] \text{NEM}$).



Figure 3.
Illustration of mini-scale purification using a vacuum manifold.

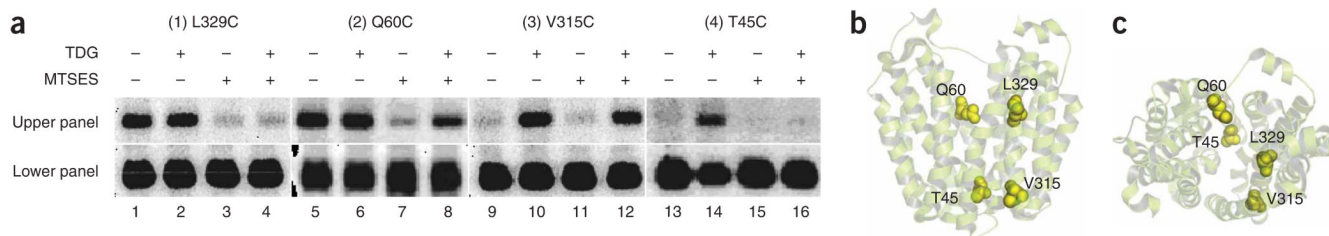


Figure 4.

Accessibility of single-Cys LacY mutants to methanethiosulfonate ethylsulfonate (MTSES) and effect of ligand. **(a)** Right-side out (RSO) membrane vesicles [approximately 1 mg protein in 50 μ l 100 mM KP_i (pH 7.5)/10 mM MgSO₄] prepared from *Escherichia coli* T184 and transformed with plasmid encoding the indicated single-Cys LacY were incubated without or with MTSES (0.2 mM final concentration) for 5 min at 25 °C in the absence or presence of β -D-galactopyranosyl 1-thio- β -D-galactopyranoside (TDG), as indicated. Vesicles were washed two times with ice-cold buffer and resuspended in 50 μ l of the same buffer and TDG (10 mM final concentration) was added back to the samples initially treated with TDG. Samples were then treated with *N*-[ethyl-1-¹⁴C]ethylmaleimide ([¹⁴C]NEM) (40 mCi mmol⁻¹; 0.5 mM final concentration) for 30 min at 25 °C. Reactions were quenched with DTT, and biotinylated LacY was solubilized and purified as described in the protocol. Aliquots containing approximately 5 μ g protein were separated by SDS/12% polyacrylamide gel electrophoresis (PAGE). The gel was dried and exposed to a PhosphoImager screen for 5–8 d. Incorporation of [¹⁴C]NEM (upper panel) was visualized and quantitated by a Storm 860 PhosphoImager (Molecular Dynamics). A fraction of the protein (0.5 μ g) eluted from avidin gel was analyzed by western blotting with anti-C-terminal antibody (lower panel). **(b)** Mapping of the residues in the x-ray crystal structure of LacY (side view). **(c)** Cytoplasmic view.

TABLE 1

Troubleshooting table

Problem	Possible reason	Solution
No protein band is present as probed with western blot and no radioactive band is visible	1 Immobilized monomeric avidin gel was not pretreated with biotin	1 Treat immobilized monomeric avidin gel with 3 mM biotin using a packed column; do not use the <i>batch method</i>
	2 Elution buffer did not contain biotin	2 Add biotin to elution buffer
	3 Elution buffer did not contain the proper concentration of detergent	3 Add detergent to elution buffer
	4 The sample was heated before SDS-polyacrylamide gel electrophoresis(SDS-PAGE)	4 Make sure not to heat the sample before running SDS-PAGE
A weak protein band detected using the PhosphoImager	Low level of expression	Increase level of membrane protein expression or expose the gel to the PhosphoImager for longer
Problematic extraction of N -[ethyl- ^{14}C]ethylmaleimide from pentane	Pentane may react with certain plastic tubes	Use glass tubes
Partially exposed image of the labeled protein band	Cassette not closed completely (partly exposed to light)	Close the cassette tightly
Presence of superimposed images	Failure to erase storage phosphor screen	Clean well and expose the screen for longer time