Chapter 2

Practical Aspects in Expression and Purification of Membrane Proteins for Structural Analysis

Kutti R. Vinothkumar, Patricia C. Edwards, and Joerg Standfuss

Abstract

A surge of membrane protein structures in the last few years can be attributed to advances in technologies starting at the level of genomes, to highly efficient expression systems, stabilizing conformational flexibility, automation of crystallization and data collection for screening large numbers of crystals and the microfocus beam lines at synchrotrons. The substantial medical importance of many membrane proteins provides a strong incentive to understand them at the molecular level. It is becoming obvious that the major bottleneck in many of the membrane projects is obtaining sufficient amount of stable functional proteins in a detergent micelle for structural studies. Naturally, large effort has been spent on optimizing and advancing multiple expression systems and purification strategies that have started to yield sufficient protein and structures. We describe in this chapter protocols to refold membrane proteins from inclusion bodies, purification from inner membranes of *Escherichia coli* and from mammalian cell lines.

Key words: Expression systems, *Escherichia coli*, Inclusion bodies, HEK cells, Rhodopsin

1. Introduction

The primary requirement of any successful structural project is a necessity for abundant source material. Only a handful of membrane proteins are found in such high amounts so that they can be directly isolated from the native membranes (1). Most membrane proteins are expressed in low abundance hence there is a need to identify an optimal expression system that produces sufficient material for structure determination but at the same time preserving their function. Due to their inherent hydrophobic nature, over-expression of membrane proteins is not straightforward and often results in proteins targeted to the wrong cellular compartments, misfolded

Ingeborg Schmidt-Krey and Yifan Cheng (eds.), *Electron Crystallography of Soluble and Membrane Proteins: Methods and Protocols*, Methods in Molecular Biology, vol. 955, DOI 10.1007/978-1-62703-176-9_2,

and therefore not functional and/or degraded (2). Over the years several different heterologous over-expression systems have been identified facilitating structure determination of a large variety of membrane proteins obtained from bacterial, yeast, insect, and human cells (3). A recent addition includes cell-free translation of membrane proteins that has shown promise in obtaining functional proteins (4) and in one case structure (5). We would like to recommend the readers the following reviews (3, 6–8) and book chapters in Methods in Molecular Biology (9) for in depth description of different expression systems available for membrane proteins.

The process to obtain a good quality protein for structural studies can be divided into two steps: (1) finding a suitable host for expression and (2) extraction of protein from cellular membranes and subsequent purification. The three examples of membrane proteins described here include protein expressed as inclusion bodies or in the inner membranes of *Escherichia coli* and in HEK cells that have resulted in crystals and eventually structure (10–14). Expression of proteins as inclusion bodies results in large yield, however a need to refold often limits the final amount of protein for structural analysis. Structures of a number of β-barrel membrane proteins refolded from inclusion bodies have been successfully determined (15). There are also numerous examples of α-helical membrane proteins that have been refolded from inclusion bodies and shown to be functional but their structures yet to be determined (16–19). The expression into *E. coli* inner membranes has so far been the most successful for membrane proteins from prokaryotic source, and the relative ease with which huge quantity of protein can be produced makes it still the most attractive system. Recent success in determining structure of eukaryotic membrane proteins with material obtained from COS or HEK cells are promising (13, 14, 20). These include the well-studied bovine rhodopsin and trimeric Rh protein of the ammonia transporter family. Although rhodopsin can be isolated in abundance from bovine retinas, the advantage of over-expression can be exemplified with the ability to make mutants that are medically relevant or that stabilize a particular conformation (27). The procedures described here is applicable to any membrane protein of interest since most proteins upon extraction from membranes are enriched using specific affinity tags, however depending on the expression system certain modifications need to be made or depending on protein additional steps/factors may be required for purification.

2. Materials

2.1. Protein Expression

1. Expression vectors: OmpG without leader sequence or an affinity tag is expressed from a T7 promotor in a pET26b vector (see Note 1). GlpG is expressed with a C-terminal his

tag in pET25b vector. Rhodopsin is expressed in pACMVtetO vector (21).

2. Strains: *E. coli* C41 or C43 (see Note 2).
   Human embryonic kidney (HEK) cells (HEK-293S-GnTI$^-$, a mutant cell line for restricted and homogenous glycosylation) (22).
3. Antibiotics: The stock solutions of antibiotics are ampicillin (100 mg/mL), kanamycin (100 mg/mL), blasticidin (5 mg/mL), geneticin (50 mg/mL), tetracycline (2 mg/mL). Tetracycline stock is made in ethanol, the rest of the antibiotics can be dissolved in water.
   Final concentrations of antibiotics used: ampicillin (100 μg/mL), kanamycin (50 μg/mL), blasticidin (5 μg/mL), geneticin –G418 (200 μg/mL), tetracycline (2 μg/mL).
4. Media for expression in *E. coli*:
   LB medium:
   10 g Bacto tryptone.
   5 g Bacto yeast extract.
   5 g NaCl.

   2XYT:
   16 g Bacto tryptone.
   10 g Bacto yeast extract.
   5 g NaCl.
   2 g Glucose.
5. Media for propagation and expression in HEK-293S cells:
   Dulbecco's modified Eagle's medium/F12 (DMEM) supplemented with 10% fetal calf serum (FCS), PenStrep (Gibco), Geneticin-G418 and blasticidin.
   Freestyle medium (Invitrogen) supplemented with 5% FCS and PenStrep. (Alternatively, Protein expression medium from Gibco with 10% newborn calf serum, NBCS can be used. NBCS is more cost effective).
   IPTG (isopropyl-β-d-1-thiogalactopyranoside):
   A 1 M stock is prepared in water and stored at –20°C.
6. Detergents:
   20% stock solutions of detergents *n*-Octyl-β-d-glucopyranoside, *n*-Nonyl-β-d-glucopyranoside, *n*-Decyl-β-d-maltopyranoside (from Anatrace) are prepared and stored at –20°C. Triton X-100 is obtained from Sigma.
7. Buffers and reagents:
   1M Tris–HCl pH 8, 1 M HEPES pH 7, 1 M glycine pH 8, 1 M sodium bicarbonate pH 8.5, 1 M Mes pH 5.0, 1 M ammonium bicarbonate, 1 M sodium acetate pH 4.0, 5 M sodium chloride, 1 M sodium azide, 1 M Tris-HCl pH 6.8, Phosphate-buffered saline (PBS), chymotrypsin (Sigma), Bradford reagent (Sigma), sodium butyrate (Sigma, made fresh each time

before use), 1D4, known as GC R1, antibody is bought from the University of British Columbia. http://rho1D4.com, 11-cis retinal (a gift from Rosalie Crouch, which is dissolved in ethanol. See also Note 16).

8. Lysis buffer: 25 mM Tris–HCl, pH 8 with Roche complete protease inhibitor cocktail (four tablets for 200 mL).
9. Urea solution:
   Fresh solution of urea is prepared just before the start of purification. Typically 1–2 L of 8 M stock is prepared.
10. Transfection: Lipofectamine 2000 obtained from Invitrogen.
11. Chromatography materials: DEAE sepharose fast flow, Q-sepharose fast flow, CNBR-activated sepharose 4B, Superdex 200 (10/300 GL) obtained from GE healthcare.

2.2. Protein Analysis

1. Protein gel electrophoresis system (Invitrogen) (see Note 3).
2. 4× protein sample buffer (0.24 M Tris–HCl, pH 6.8, 8% Sodium dodecyl sulfate (SDS), 40% glycerol, 0.04% (w/v) bromophenol blue, 5% 2-mercaptoethanol).
3. Running buffer (see Note 4).
4. Molecular weight standards (SIGMA wide range marker or equivalent).
5. Gel staining solution (Coomassie blue R250 staining solution in 50% methanol, 10% acetic acid).
6. Destaining solution (10% (v/v) ethanol, 10% (v/v) acetic acid).

3. Methods

3.1. OmpG

3.1.1. Protein Expression

1. Transform BL21 (DE3) C41 competent cells with OmpG plasmid, plate cells on LB agar plates with kanamycin and incubate overnight at 37°C.
2. Inoculate a single colony in 25 mL LB medium containing kanamycin in a 250 mL flask and grow for 12–16 h at 37°C with shaking (200 rpm).
3. Dilute the culture into 2 L of 2XYT medium with kanamycin in a 5 L flask and incubate at 37°C with shaking (180 rpm).
4. At an OD600 of 0.6, add 0.5 mM IPTG and allow cell growth for 3–4 h.
5. Harvest the cells by centrifugation (4,000×*g* for 10 min at 4°C).

3.1.2. Cell Disruption and Harvesting of Inclusion Bodies

1. Resuspend the cells in 50 mL of lysis buffer.
2. Break the cells by two passages through an emulsiflex (see Note 5) at 15,000 kpi.

3. Collect inclusion bodies (IB) by centrifugation for 20 min at 6,000 × *g* at 4°C.
4. Wash IB in 25 mM Tris–HCl, pH 8, 1 M urea, and 1% Triton-X 100 and centrifuge for 20 min at 6,000 × *g* at 4°C.
5. Resuspend IB in 25 mM Tris–HCl, pH 8 and 8 M urea (see Note 6).

3.1.3. Purification and Refolding of OmpG

1. Equilibrate 10 mL of DEAE sepharose fast flow with 25 mM Tris–HCl, pH 8 and 8 M urea (urea buffer).
2. Apply IB to DEAE column and wash extensively with 150 mL urea buffer.
3. Remove weakly bound protein by washing the column with 50 mL of urea buffer containing 50 mM and 165 mM NaCl in successive steps.
4. Elute OmpG with a buffer containing 300 mM NaCl in 25 mM Tris–HCl, pH 8 and 8 M urea.
5. Dilute OmpG in 75 mM *n*-octyl glucoside (OG) such that the final concentration of urea is ~3 M and final protein concentration is ~0.4 mg/mL (see Note 7).
6. Incubate the diluted mixture at RT for 12–16 h.
7. Load 5–10 µL of refolded OmpG in a SDS-PAGE gel and monitor the efficiency of refolding (see Note 8 and Fig. 1a).
8. Urea is removed by dialysis against a buffer with 25 mM Tris–HCl, pH 8 and 25 mM OG.
9. Alternatively, refolded OmpG can be loaded onto an anion-exchange column and urea removed (see Note 9), this enriches the folded product. Refolded OmpG can then be reconstituted back into lipid bilayers to obtain 2D crystals (Fig. 1b, c) or for 3D crystals (10).

3.2. Expression and Purification of GlpG

3.2.1. Protein Expression

1. Transform BL21 (DE3) C41 competent cells with GlpG plasmid, plate cells on LB agar plates with ampicillin and incubate overnight at 37°C.
2. Inoculate a single colony in 5 mL LB medium containing ampicillin in a 20 mL glass tube and grow for 8–10 h at 37°C with shaking (200 rpm).
3. Dilute the culture into 300 mL of LB medium with ampicillin in a 1 L flask and incubate at 37°C with shaking (200 rpm).
4. Dilute 25 mL of cells in 1 L 2XYT medium with ampicillin in a 2 L flask and incubate at 37°C with shaking (200 rpm).
5. At an OD600 of 0.6, add 0.5 mM IPTG. At this point, reduce the growth temperature to 24°C and allow the cells to grow overnight.
6. Harvest cells by centrifugation (4,000 × *g* for 10 min at 4°C).

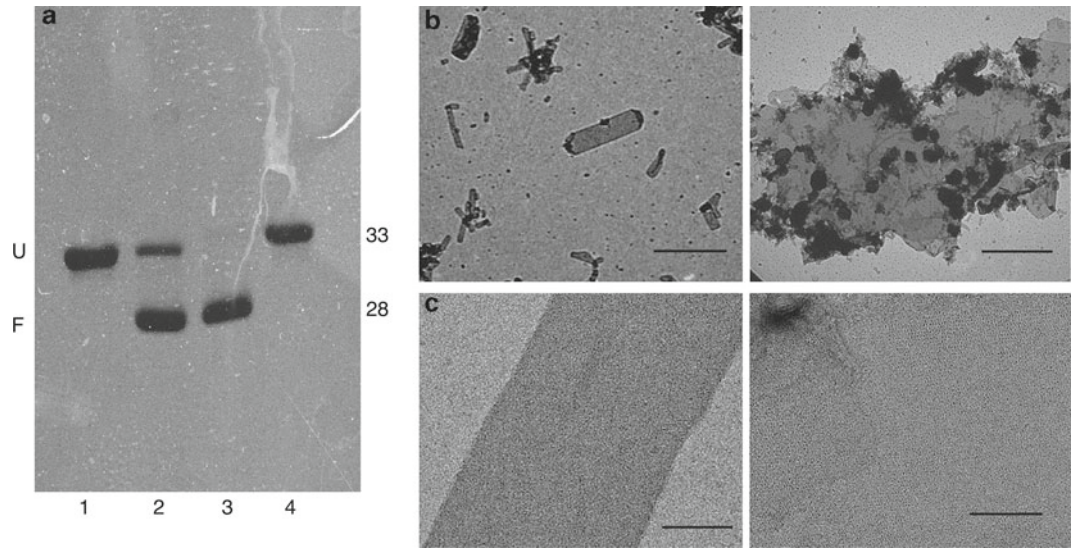

Fig. 1. (**a**) OmpG purified and refolded from inclusion bodies: A SDS PAGE gel showing the differential migratory pattern of OmpG. *Lane 1* shows OmpG in 8 M urea and migrates at ~33 kDa. *Lane 2* shows a mixture obtained after dilution of OmpG in 75 mM Octyl glucoside and removal of urea, with the folded species migrating faster at ~28 kDa. A further enrichment in an ion-exchange column results in removal of unfolded protein as shown in *lane 3*. The same sample when heated at 95°C for 5 min prior to loading in the gel results in complete denaturation of OmpG as shown in *lane 4*. Equal amount of protein was loaded in all lanes. (**b**, **c**) Two-dimensional (2D) crystals of refolded OmpG: Refolded OmpG in detergent is mixed with lipids and gradual removal of detergent by dialysis results in the formation of 2D crystals. Panel (**b**) shows low magnification CCD images of negatively stained 2D crystals of OmpG with two distinct morphologies either as isolated tubes or stacked membranes (scale bar—1 μM). Panel (**c**) shows the crystal lattice of these crystals as viewed in higher magnification (scale bar—100 nm).

3.2.2. Cell Disruption and Preparation of Membrane Fraction

1. Resuspend cells in 250 mL of lysis buffer and homogenize completely.
2. Break the cells by two passages through an emulsiflex (see Note 5) at 15,000–20,000 kpi.
3. Remove unbroken cells by centrifugation for 20 min at 6,000 × *g* at 4°C.
4. Distribute the supernatant into polypropylene tubes suitable for Ti45 rotors (Beckmann) and collect the membrane fraction by centrifugation at 100,000 × *g* at 4°C for 120 min.
5. Resuspend the membrane fraction in 25 mM Tris–HCl, pH 8 and estimate the total protein concentration (see Note 10).
6. Aliquot the membranes at a concentration of ~40–50 mg/mL and store at −80°C.

3.2.3. Solubilization and Purification of GlpG

1. Thaw the membranes in a warm water bath. Dilute the membranes with 25 mM Tris–HCl, pH 8 to a final concentration of 5 mg/mL (see Note 10).
2. Add *n*-β-decyl-maltoside (DM) from a stock of 20% to a final concentration of 1.25% to initiate the solubilization.

3. Allow membrane solubilization at RT for 30–45 min with continuous stirring.
4. Separate the unsoluble fractions by ultracentrifugation at 100,000 × *g* for 30 min at 4°C.
5. Pre-equilibrate 4–5 mL of Ni-NTA column with DM buffer (25 mM Tris pH 8, 0.3 M NaCl, 0.2% DM) containing 10 mM imidazole (see Note 11).
6. Add imidazole and NaCl to final concentrations of 10 mM and 0.3 M, respectively, to the detergent soluble fraction (see Note 12).
7. Pass the supernatant through the Ni-NTA column and collect the unbound fraction by gravity flow.
8. Wash the Column with 20× DM buffer containing 10 and 30 mM imidazole in succession to remove nonspecifically bound proteins (see Note 12).
9. Elute GlpG with 0.2 M imidazole in DM buffer and estimate the protein concentration with Bradford (23) reagent (Sigma).
10. Add chymotrypsin (Sigma) at a ratio of 1:50 (w/w) and incubate at RT for 36 h to remove the soluble N-terminal domain of GlpG (see Note 13).
11. Exchange buffer to remove salts by concentration and dilution on a Vivaspin concentrator with a 10 kDa cutoff.
12. Pass the concentrated protein through Q-sepharose fast flow column pre-equilibrated with 25 mM Tris–HCl, pH 8 and 0.2% DM.
13. Collect the flow through, which contains the majority of N-terminally truncated GlpG.
14. Concentrate the protein to 0.5 mL and load on to Superdex-200 column pre-equilibrated with 0.5% *n*-nonyl-β-glucoside.
15. Collect peak fractions (~13 mL) of GlpG and concentrate with a Vivaspin concentrator with a 10 kDa cutoff. This procedure yields ~0.5–1 mg of truncated GlpG from a liter of culture and both 2D (Fig. 2) and 3D crystals can be obtained (11, 12).

3.3. Rhodopsin

3.3.1. Preparation of Stable Cell Lines

1. Grow HEK293-GnTI-cells (21) in DMEM/F12 medium supplemented with 10% FCS, blasticidin, at 37°C in an environment with 5% CO_2 (see Note 14).
2. Day 1; split the cells into a 10 cm plate with medium free of antibiotic and plate cells such that they will attain 90% confluency for transfection, the next day.
3. Day 2; mix rhodopsin DNA (20 μg per transfection) gently with 1.5 mL serum and antibiotic-free media. Add 60 μL Lipofectamine 2000 separately to 1.5 mL serum-free media and leave at RT for 5 min. Combine the DNA and Lipofectamine

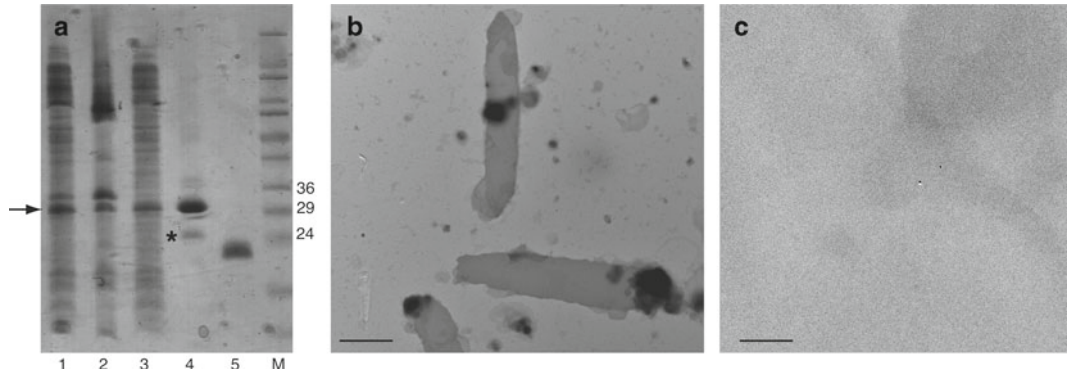

Fig. 2. (**a**) Purification of GlpG from inner membrane of *E. coli*: GlpG, a rhomboid protease is homologously expressed in *E. coli* in the inner membranes. *Lanes 1–3* show the total membrane proteins, unsoluble fraction after detergent extraction and proteins extracted by detergent, respectively. Equal amount of protein (15 μg) was loaded. The band marked with *arrow* shows the GlpG full-length protein. A prominent band in the membrane fraction when visualized by Coomassie stain is a good indicator of efficient expression. A comparison of the unsoluble and soluble fractions shows that >80% of GlpG has been extracted by decyl maltoside. In *lane 4*, GlpG purified using a his-tag in decyl maltoside but left at 4°C for 2 days shows the appearance of a proteolysed product (*marked with asterisk*). *Lane 5* shows the N-terminally truncated GlpG after ion exchange and gel filtration columns. *Lane M* denotes the sigma wide range marker used in this gel. (**b**, **c**) Two-dimensional crystals of GlpG: N-terminally truncated GlpG in detergent was mixed with *E. coli* polar lipids and detergent removal by dialysis results in 2D crystals (12). Panel (**b**) shows a low magnification view of negatively stained GlpG 2D crystals (scale bar—2 μM). Panel (**c**) shows the crystal lattice as viewed in higher magnification (scale bar—100 nm). Panels (**b**) and (**c**) are reproduced with permission from ref. 12.

together, mix gently, and leave for 20 min. Add this mixture in drops to the plate of cells and rock the plate back and forth to spread the mixture.

4. Day 3; split the cells tenfold into media containing blasticidin.
5. Day 4; replace media with DMEM/F12 containing blasticidin and geneticin (G418) (250 μg/mL), and 20% conditioned media (see Note 15).
6. Cells transfected with rhodopsin survive in the presence of G418 and start to grow. After ~2 weeks, small colonies can be seen growing. At this stage, these colonies can be picked clonally or all cells surviving G418 selection can be harvested and expanded.

3.3.2. Expression in HEK Cells

1. Expand cells containing rhodopsin under tetracycline inducible promotor as adherent cells to confluency.
2. Collect the confluent cells, count and seed in Freestyle media (Invitrogen) plus 5% FCS at 0.5×10^6 cells/mL. Typically, cells from five 75-cm^2 flasks are used to expand to a 300 mL suspension culture. Cells stay in suspension rotating on an orbital shaker at 125 rpm.

3. After 2–3 days this initial suspension culture is diluted into ~1 L media resulting in 0.5×10^6 cells/mL in a Wave bioreactor (GE Healthcare). The bag is supplied with 8% CO_2. The rock rate rises between 10 and 25 as the bag is filled, while the angle rises from 5 to 7.
4. Allow cells to grow to a density of ~2×10^6 cells/mL and dilute to 0.5×10^6 cells/mL every 2–3 days with Freestyle medium plus 5% FCS.
5. At 9 L induce rhodopsin expression by adding a final concentration of tetracycline (2 μg/mL) and sodium butyrate (5 mM) diluted in 1 L of medium.
6. Allow cell growth for 72 h after induction, cell density is typically 2–3×10^6 cells/mL.
7. Harvest the cells by centrifugation at $3{,}000 \times g$ for 10 min at 4°C.
8. Wash cell pellets with PBS buffer containing protease inhibitor cocktail.
9. Freeze cell pellets in liquid nitrogen and store at –80°C.

3.3.3. Preparation of 1D4 Column

1. 1D4 is dialyzed against three changes of 5 mM NH_4HCO_3 and then lyophilized (optional).
2. To 1 g of CNBr-activated sepharose 4B, add 50 mL of 1 mM HCl. Leave rotating until the column material has swelled and is in homogeneous suspension. 1 g should swell to 3.5 mL in a few minutes.
3. Wash the resin with 50 mL of 1 mM HCl.
4. Wash with 50 mL 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5.
5. Dissolve antibody in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5.
6. Add 14 mg antibody to 3.5 mL of sepharose. Leave over night at 4°C.
7. Wash with 50 mL 0.2 M glycine, pH 8.0.
8. Incubate in 50 mL 0.2 M glycine, pH 8.0 at RT for 2 h.
9. Wash with 50 mL 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5.
10. Wash with 50 mL 0.1 M NaOAc, 0.5 M NaCl, pH 4.0.
11. Wash with 50 mL 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5.
12. Wash with PBS/10 mM sodium azide and store in the same solution at 4°C.

3.3.4. Purification of Rhodopsin and Reconstitution with Retinal

1. Thaw cell pellets and resuspend in PBS buffer containing protease inhibitor cocktail.
2. Initiate solubilization by the addition of 1.25% DM and incubate for 1 h at 4°C.

3. Centrifuge at 100,000 × *g* at 4°C for 30 min to remove insoluble fractions.
4. Incubate supernatant with 1D4 antibody column pre-equilibrated with PBS containing DM (0.125%) for 2–4 h at 4°C.
5. Wash the matrix with PBS/0.125% DM. All subsequent steps are done in dim red light.
6. At this point, add 50 μM 11-cis retinal to the matrix and incubate overnight at 4°C (see Note 16).
7. Wash the matrix with PBS and 0.125% DM to remove excess retinal.
8. Protein is exchanged to detergent OG (1%) in 10 mM HEPES, pH 7.0 (see Note 17).
9. Elute rhodopsin with peptide TETSQVAPA (80 μM) resembling the C-terminus of rhodopsin, in 10 mM HEPES pH 7 and 1% OG buffer. Repeat elution three times.
10. Concentrate the protein to 0.5 mL with a Vivapsin 30 kDa cut-off concentrator.
11. Load reconstituted recombinant rhodopsin onto a Superdex-200 column pre-equilibrated with 10 mM Mes pH 5.0, 0.1 M NaCl and 1% OG.
12. Collect peak fractions and concentrate to 10–15 mg/mL for crystallization trials. Typically, 0.2–0.4 mg of recombinant rhodopsin with correctly bound retinal and homogenous glycosylation (Fig. 3) can be obtained from a liter of suspension culture.

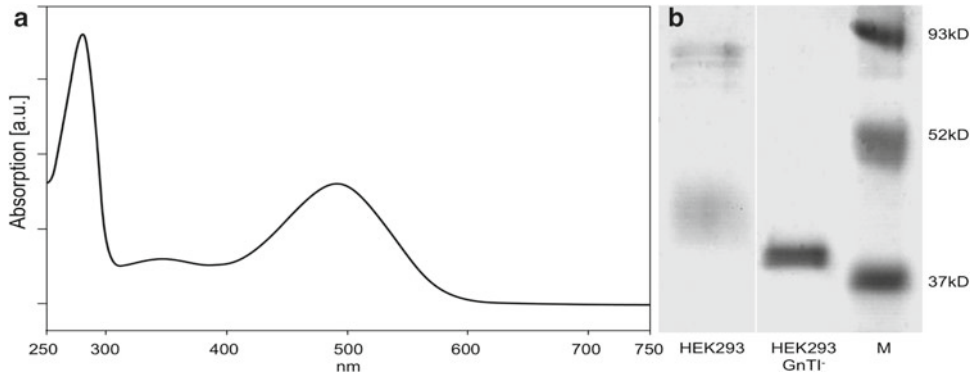

Fig. 3. (**a**) The UV-VIS absorption spectrum of purified recombinant rhodopsin shows the characteristic peak at 280 nm from protein and that at 500 nm from the protonated 11-*cis*-retinal ligand. The ratio of these two peaks is often used to access the quality of a rhodopsin preparation as misfolded protein fails to bind retinal and increases the relative 280 nm contribution. HEK293-GnTI⁻ cell expressed rhodopsin routinely shows a ratio that is very close to the 1.6 ratio observed for native rhodopsin purified from bovine retinas indicating that nearly all protein expressed are in a functional form. (**b**) SDS PAGE comparing rhodopsin expressed in HEK293S and HEK293S-GnTI⁻ cells. The homogenous glycosylation of HEK293S-GnTI⁻ cell expressed rhodopsin leads to a faster and more even migration compared to the complex glycosylation pattern of HEK293S cell expressed protein.

4. Notes

1. For expression in *E. coli*, good expression is typically obtained with any of the pET vectors available from Novagen. The choice of the tags depends on protein and the best option is identified by trials. Use of T7 promoter (as is the case with most pET vectors) sometimes is detrimental to expression with protein ending in inclusion bodies. As an alternative, controlled expression can be achieved with arabinose inducible pBAD system or similar other vectors. For a detailed list of vectors and strains, please refer to Junge et al. 2008 and Zoonens & Miroux 2010 (28).
2. We have had greater success with BL21 derivatives C41 and C43 than the parent strains as explained by Miroux and Walker (24). However, some membrane proteins do not express at high levels, which could be due to codon usage or inefficient insertion into membranes.
3. Invitrogen offers a range of gels and buffer systems. Bis-tris gels in combination with Mes buffer is the most commonly used system. However, we have observed for many membrane proteins (in particular those that are very hydrophobic) the best results are achieved with a gradient gel run with Tris-glycine buffer.
4. Membrane proteins are not typically heated prior to loading in a gel, as they tend to aggregate (beta-barrel membrane proteins can be heated, see Note 8). Since they are not completely denatured by SDS, the ratio of SDS bound to the protein differs from that of a soluble protein. This results in faster migration of membrane proteins typically 4–8 kDa lower than the size estimated from the amino acid sequence.
5. Lysis of *E. coli* cells and preparation of the membrane fraction is a critical step to remove unfolded proteins. When compared to yeast, *E. coli* cells are easily and efficiently disrupted using high-pressure systems such as Emulsiflex C3/C5 from Avestin or Constant cell disruption system.
6. Guanidium hydrochloride (GuHCl) at a concentration of 6 M can be used instead of urea. Remember to dialyze out GuHCl prior to loading in a gel.
7. The critical micelle concentration (CMC) of detergent micelles differs depending on environment such as ionic strength, additives, and temperature. For instance, the CMC of octyl glucoside in water is 25 mM but in 8 M urea the CMC increases to 42.5 mM. Thus it is important to take into account the concentration of urea when attempting to refold a membrane protein.

8. Beta-barrel membrane proteins show different migratory behavior on SDS PAGE gel depending on whether the sample is heated. When boiled at 95°C for 5 min, beta-barrel membrane proteins are completely denatured and the mobility closely matches that of the estimated mass from the amino acid sequence. If the sample is not boiled, the protein migrates faster. This difference in mobility can be used to monitor the efficiency of refolding.
9. Different approaches can be utilized for refolding that include gradual dilution of denaturant in an appropriate refolding buffer, exchange of buffer by dialysis or refolding on the column. Success depends on the protein of choice. We found that gradual dilution worked best for OmpG in terms of the efficiency and yield of refolded protein. A database comprising details of refolding of various proteins can be found at http://refold.med.monash.edu.au.
10. Estimation of total protein concentration in the membranes is a critical step, since it determines the amount of detergent to be used for solubilization. Use of less detergent as a result of underestimating the protein concentration can result in inefficient extraction of protein from membranes. A modified Lowry's protocol that uses acid precipitation and detergent gives a reliable estimate (25).
11. Application of detergent soluble fraction to Ni-NTA resin can be performed by batch method or directly on the column. The source of Ni-NTA can sometimes be crucial on the yield of the protein. We use Ni-NTA from Qiagen and regenerate it to be used multiple times.
12. Addition of imidazole and sodium chloride is optional and depends on efficiency of protein binding to the resin. Use of low concentration of imidazole in the binding step is beneficial in preventing nonspecific binding. The concentration of imidazole required for washing weakly bound proteins and for elution of target protein largely depends on protein of interest. As an initial step, a gradient of imidazole can be used to determine these concentrations. AcrB, a multi-drug transporter is a very common contaminant from *E. coli* that binds to Ni-NTA with very high affinity. Care must be taken to remove AcrB in subsequent steps.
13. In the case of GlpG, removal of N-terminal domain by a protease is a crucial step for obtaining well-diffracting crystals. An expression construct devoid of the N-terminal domain produces crystals that diffract poorly.
14. Heterogenous glycosylation of proteins expressed in eukaryotic cells is often detrimental in obtaining good crystals. With rhodopsin it was essential to use this cell line (HEK-293S-GnTI$^-$)

in combination with stabilizing mutations to obtain well-diffracting crystals (14). In some cases it may however be necessary to remove glycosylation once the protein is correctly folded to facilitate crystal growth. In such cases enzymatic digestion of HEK-293S-GnTI$^-$ expressed proteins with EndO H or PNGase F has been used very successfully (26).

15. Conditioned media is harvested from near confluent cells and filtered that contains conditioning factors. Media is stored at –20°C and used for selection of stably transfected cell lines.
16. Synthetic 9-cis retinal (Sigma) can be used instead of 11-cis retinal. Both these ligands are inverse agonists of rhodopsin with similar potency.
17. Exchange of detergent to octyl glucoside and addition of lipids is a crucial step to obtain diffracting crystals of recombinant rhodopsin (14).

Acknowledgments

VK acknowledges the European Union for a Marie-Curie Intra-European Fellowship and the Medical Research Council for a Career Development fellowship. The work on OmpG was done by VK at the Max-Planck Institute of Biophysics, Frankfurt as part of Ph.D. thesis. This work was further supported by the Swiss National Science Foundation (SNSF) grants 31003A_132815 and 31003A_141235 to JS. We thank Gebhard Schertler at the PSI and Daniel Oprian at the Brandeis University for discussion and advice on the expression of rhodopsin.

References

1. Vinothkumar KR, Henderson R (2010) Structures of membrane proteins. Q Rev Biophys 43:65–158
2. Tate CG, Haase J, Baker C, Boorsma M, Magnani F, Vallis Y, Williams DC (2003) Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter. Biochim Biophys Acta 1610:141–153
3. Junge F, Schneider B, Reckel S, Schwarz D, Dotsch V, Bernhard F (2008) Large-scale production of functional membrane proteins. Cell Mol Life Sci 65:1729–1755
4. Junge F, Haberstock S, Roos C, Stefer S, Proverbio D, Dotsch V, Bernhard F (2010) Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins. N Biotechnol 28:262–271
5. Chen YJ, Pornillos O, Lieu S, Ma C, Chen AP, Chang G (2007) X-ray structure of EmrE supports dual topology model. Proc Natl Acad Sci USA 104:18999–19004
6. Sorensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol 115:113–128
7. Midgett CR, Madden DR (2007) Breaking the bottleneck: eukaryotic membrane protein expression for high-resolution structural studies. J Struct Biol 160:265–274
8. Lee JK, Stroud RM (2010) Unlocking the eukaryotic membrane protein structural proteome. Curr Opin Struct Biol 20:464–470
9. Mus-Veteau I (2010) Heterologous expression of membrane proteins for structural analysis. Methods Mol Biol 601:1–16

10. Yildiz O, Vinothkumar KR, Goswami P, Kuhlbrandt W (2006) Structure of the monomeric outer-membrane porin OmpG in the open and closed conformation. EMBO J 25: 3702–3713
11. Wang Y, Zhang Y, Ha Y (2006) Crystal structure of a rhomboid family intramembrane protease. Nature 444:179–180
12. Vinothkumar KR (2011) Structure of rhomboid protease in a lipid environment. J Mol Biol 407:232–247
13. Standfuss J, Xie G, Edwards PC, Burghammer M, Oprian DD, Schertler GF (2007) Crystal structure of a thermally stable rhodopsin mutant. J Mol Biol 372:1179–1188
14. Standfuss J, Edwards PC, D'Antona A, Fransen M, Xie G, Oprian DD, Schertler GF (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. Nature 471:656–660
15. Hiller S, Abramson J, Mannella C, Wagner G, Zeth K (2010) The 3D structures of VDAC represent a native conformation. Trends Biochem Sci 35:514–521
16. Standfuss J, Kuhlbrandt W (2004) The three isoforms of the light-harvesting complex II: spectroscopic features, trimer formation, and functional roles. J Biol Chem 279:36884–36891
17. Baneres JL, Martin A, Hullot P, Girard JP, Rossi JC, Parello J (2003) Structure-based analysis of GPCR function: conformational adaptation of both agonist and receptor upon leukotriene B4 binding to recombinant BLT1. J Mol Biol 329:801–814
18. Baneres JL, Mesnier D, Martin A, Joubert L, Dumuis A, Bockaert J (2005) Molecular characterization of a purified 5-HT4 receptor: a structural basis for drug efficacy. J Biol Chem 280:20253–20260
19. Park SH, Prytulla S, De Angelis AA, Brown JM, Kiefer H, Opella SJ (2006) High-resolution NMR spectroscopy of a GPCR in aligned bicelles. J Am Chem Soc 128:7402–7403
20. Gruswitz F, Chaudhary S, Ho JD, Schlessinger A, Pezeshki B, Ho CM, Sali A, Westhoff CM, Stroud RM (2010) Function of human Rh based on structure of RhCG at 2.1 A. Proc Natl Acad Sci USA 107:9638–9643
21. Reeves PJ, Kim JM, Khorana HG (2002) Structure and function in rhodopsin: a tetracycline-inducible system in stable mammalian cell lines for high-level expression of opsin mutants. Proc Natl Acad Sci USA 99: 13413–13418
22. Reeves PJ, Callewaert N, Contreras R, Khorana HG (2002) Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. Proc Natl Acad Sci USA 99:13419–13424
23. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
24. Miroux B, Walker JE (1996) Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260:289–298
25. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
26. Chang VT, Crispin M, Aricescu AR, Harvey DJ, Nettleship JE, Fennelly JA, Yu C, Boles KS, Evans EJ, Stuart DI, Dwek RA, Jones EY, Owens RJ, Davis SJ (2007) Glycoprotein structural genomics: solving the glycosylation problem. Structure 15:267–273
27. Deupi X, Edwards P, Singhal A, Nickle B, Oprian D, Schertler G, Standfuss J (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II 109:119–124
28. Zoonens M, Miroux B (2010) Expression of membrane proteins at the Escherichia coli membrane for structural studies. Methods Mol Biol 601:49–66

http://www.springer.com/978-1-62703-175-2

Electron Crystallography of Soluble and Membrane Proteins
Methods and Protocols
Schmidt-Krey, I.; Cheng, Y. (Eds.)
2013, XIII, 586 p., Hardcover
ISBN: 978-1-62703-175-2
A product of Humana Press