

3.2. EXPRESSION AND PURIFICATION OF MEMBRANE PROTEINS

Table 3.2.2.1Strategies for improving recombinant membrane-protein expression in *E. coli*

Troubleshooting expression problems: if the 'first-pass' expression strategy is unsuccessful, then some changes in the expression construct, host strain or induction conditions may be helpful. The symptoms of the problem often give a clue as to how to make these corrections.

Symptom	Solution
<i>E. coli</i> colonies small after transformation into expression strain	Need tighter promoter such as <i>phoA</i> , <i>araC</i> etc.
Unprocessed signal sequence†	If none is used in native situation, remove from construct† If one is present in native situation, use an expression host co-translational signal sequence† If still unprocessed, lower translation initiation strength
Mostly high-molecular-weight aggregates	Decrease induction time
Rapid cell growth after induction with low expression levels	Increase translation initiation strength
Poor cell growth after induction with low expression levels (a) Accumulation maximum after only 1–3 h (b) Accumulation increases out to 8–10 h post induction	Decrease translation initiation rates or plasmid copy number Usually successful: (a) Re-synthesize the gene, removing rare codons and optimizing codon pairs† (b) Convert transmembrane and intracellular domain cysteines (normally reduced) to serines† (c) Try different non-DE3 lysogen strains after switching to a non-T7 promoter (i) Cytoplasmic protease deletions (ii) Periplasmic protease deletions (iii) Heat-shock <i>htpR</i> ^{TS} Sometimes successful: (a) Try different C-terminal tags such as poly-his, Flag etc.† (b) Try different temperatures of induction such as 20, 25, 30 °C

† These methods are also useful for optimizing eukaryotic gene expression.

3.2.2.2. Strategies for improving membrane-protein expression

Low expression levels are common for many membrane proteins. Before testing alternative hosts, it may be worthwhile first to screen alternative *E. coli* expression systems, such as those using the tightly controlled arabinose promoter (Guzman *et al.*, 1995). Also, other *E. coli* strains can be tested. For example, the mutant C41 and C43 'Walker' expression strains (Miroux & Walker, 1996; Wagner *et al.*, 2008) yield higher expression levels for some membrane proteins. Table 3.2.2.1 summarizes common strategies for improving the expression of membrane proteins in *E. coli*. If reasonable efforts in *E. coli* still do not yield sufficient expression, then an alternative expression host should be considered. The next most successful is yeast. Of the 22 recombinant eukaryotic membrane proteins whose structures have been solved by crystallography, half were produced using *Pichia pastoris* (seven targets), *Saccharomyces cerevisiae* (three targets) or *Saccharomyces pombe* (one target) (Raman *et al.*, 2006). Membrane proteins expressed in yeast are typically fused to an amino-terminal host signal sequence to promote proper membrane targeting. The use of higher eukaryotic hosts, such as insect and mammalian cells, is also increasing as more groups attempt to crystallize recombinant eukaryotic targets. The recent high-resolution structures of several G-protein-coupled receptors are notable examples of targets expressed successfully in insect cells (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Hanson *et al.*, 2008; Jaakola *et al.*, 2008; Warne *et al.*, 2008).

To date, there is only one report of a structure determined from cell-free synthesis of the target membrane protein (EmrE; Chen *et al.*, 2007). However, for several reasons, cell-free approaches should be explored as a complement to traditional expression methods. Firstly, the absence of a host cell negates toxicity problems for some target proteins. Secondly, many targets that do not express in cells can be produced cell-free

(Ishihara *et al.*, 2005; Klammt *et al.*, 2007; Savage *et al.*, 2007). Thirdly, these systems allow for considerable flexibility with respect to reaction conditions and components. Cell-free systems for prokaryotic and eukaryotic hosts have been described (Schwarz *et al.*, 2008), some of which function in the presence of solubilizing detergents (Ishihara *et al.*, 2005; Klammt *et al.*, 2007; Savage *et al.*, 2007) or lipids (Kuruma *et al.*, 2005; Schwarz *et al.*, 2007), facilitating proper folding and downstream purification of membrane-protein targets.

Many eukaryotic proteins have undergone extensive construct engineering to improve expression and/or crystallization outcomes. These include fusion to T4 lysozyme, mutation of glycosylation sites, deletion of potentially disordered regions and/or targeted evolution to identify conformationally stable mutants (Long *et al.*, 2005; Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007; Jaakola *et al.*, 2008; Magnani *et al.*, 2008; Serrano-Vega *et al.*, 2008; Warne *et al.*, 2009). Also worth mentioning is the observation that incorporation of an amino-terminal 'rhodopsin tag' comprising the first 20 amino acids of bovine rhodopsin (amino-acid sequence MNGTEGPNFYVFPFSNKTGVV) has been found to boost dramatically the expression levels of eukaryotic membrane targets expressed in insect and mammalian cells (Krautwurst *et al.*, 1998).

3.2.3. A consensus strategy for membrane-protein purification

3.2.3.1. General principles

The purification of an expressed integral membrane protein for structural studies typically involves four discrete steps: cell lysis, membrane isolation, detergent extraction and chromatographic separation. In this section, an evidence-based consensus strategy is outlined which aims to serve as a starting point for membrane-protein purification. Detailed protocols are outlined in Fig.

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3.2.3.1. Note that the most significant complication in this process is the need to use detergents in all steps of protein handling and chromatography. Detergents permit the extraction of target proteins from the membrane, but they must also maintain protein stability and activity by mimicking, as closely as possible, the lipid bilayer. Although many detergents do meet these criteria, relatively few are compatible with high-resolution structural studies.

3.2.3.2. Cell lysis and membrane isolation

Cellular lysis methods for the isolation of integral membrane proteins are similar to those for soluble targets. Cells over-expressing the protein of interest are re-suspended in a lysis buffer lacking detergent. Typically, high-flow/high-pressure cell-disruption devices are used, such as a continuous-flow homogenizer (EmulsiFlex, Avestin, Canada) or microfluidizer (Microfluidics Inc., Newton, Massachusetts, USA). Although these two devices achieve lysis by different mechanisms, both can disrupt the cells of almost all commonly used expression hosts, including yeast, and permit the rapid processing of large volumes. Other cell-disruption methods, such as sonication or nitrogen cavitation, can also be used for eukaryotic cells and, with slightly lower efficiency, *E. coli*. Protease inhibitors are included at this stage, although they are often not necessary in the later stages of purification. Following lysis, the membrane fraction of the cells is pelleted by ultracentrifugation.

3.2.3.3. Detergent extraction of membrane proteins

Following centrifugation, the crude-membrane pellet is re-suspended in a native lysis buffer. Detergent is then added to extract the membrane proteins from the lipid bilayer, at a molarity well above the critical micelle concentration (CMC, the concentration above which detergent molecules aggregate to form micelles). It is also more common than not that the same detergent used for solubilization is also used for subsequent purification and crystallization (Willis & Koth, 2008), albeit at a much lower concentration (typically just above the CMC). Broadly speaking, detergents fall into three main categories: charged (ionic), zwitterionic and non-charged (polar). Detergents containing no net charge are more likely to solubilize membrane proteins in their native state and are therefore more widely used for structural studies. In addition, detergents with sugar residues as head groups have proved particularly successful for the crystallization of membrane proteins, as have polyoxyethylene monoalkylethers (C_nE_m ; Prive, 2007; Newstead *et al.*, 2008). Of all the membrane-protein structures solved to date, almost 70% have used one of just six different detergents for solubilization (Newstead *et al.*, 2008; Willis & Koth, 2008); with

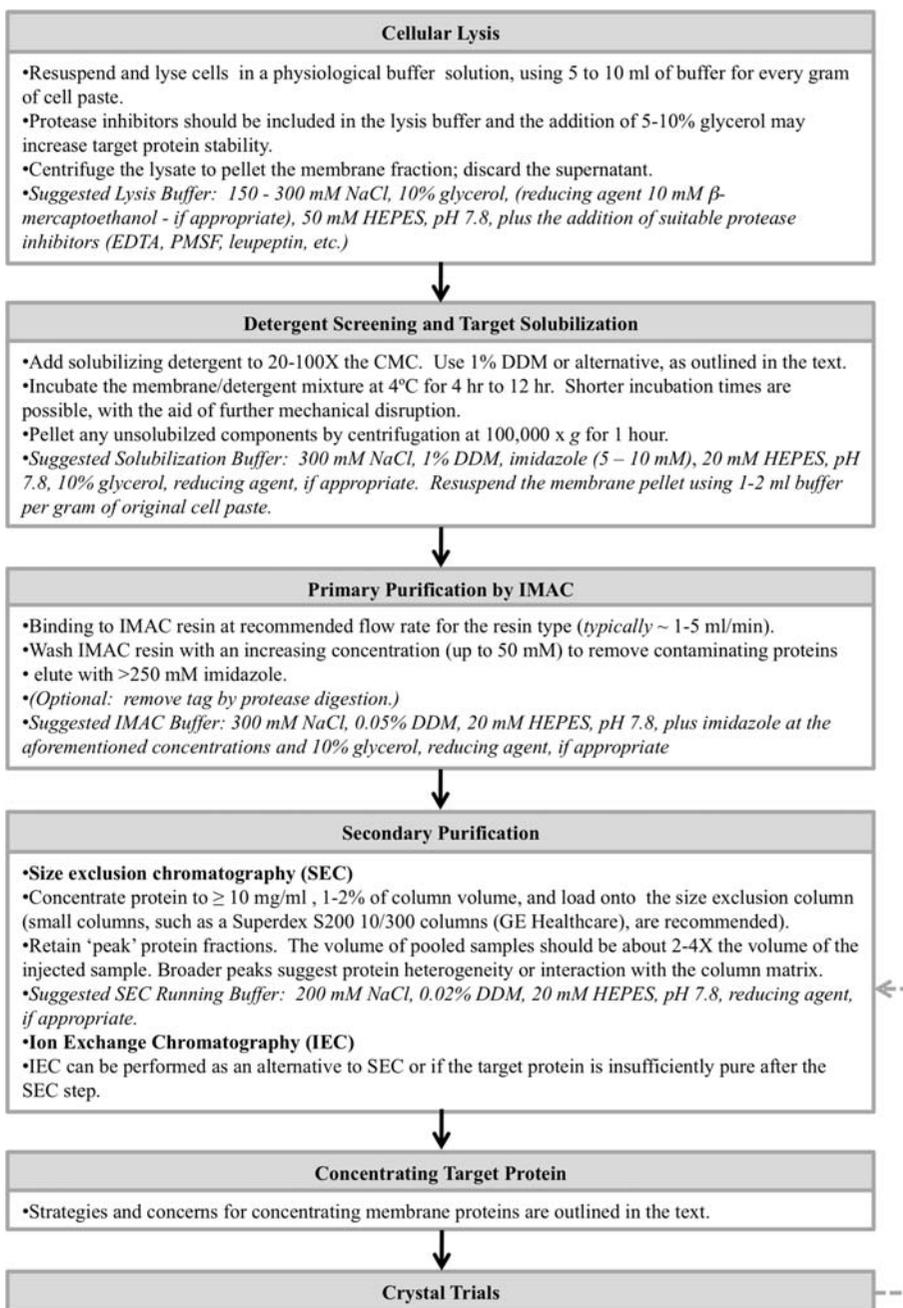


Figure 3.2.3.1

Flow diagram of the membrane-protein purification strategy. Typically, only two chromatographic steps (affinity chromatography and SEC) are required for proteins that are expressed at reasonably high levels with a hexa-histidine tag. Ion-exchange chromatography (IEC) methods are sometimes employed, although they are not as common as SEC. If no crystals are obtained in initial sparse matrix screens, the protein can be exchanged to a different detergent and crystal trials attempted again (dotted arrow). Suggested 'first-try' buffers are indicated at each step.

the exception of dodecyl-*N,N*-dimethylamine *N*-oxide (LDAO), all are non-charged. These six are, starting with the most prevalent, dodecyl- β -*D*-maltopyranoside (DDM), LDAO, octyl-polyoxyethylene (C8POE), decyl- β -*D*-maltoside (DM), octyl- β -*D*-glucoside (OG) and Triton X-100. Of these, DDM has been used for one out of almost every four crystallized targets and should be tested with any new membrane protein. The general properties of detergents and their use for membrane-protein solubilization have been reviewed by Hjelmeland (1990) and Neugebauer (1990).

The ability of detergents to solubilize target proteins can be tested on a small scale. The concentration of detergent that is optimal for solubilization should be determined empirically,

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although this can quickly become a multidimensional problem. A simple starting strategy, based on the purification methods for successfully crystallized membrane proteins, is to screen the aforementioned detergents at 1% (DDM, LDAO, Triton X-100) or 2% (C8POE, DM, OG). Detergents that are effective at these concentrations can be further screened to determine the optimal solubilization conditions (*i.e.* by varying detergent concentration *versus* solubilization time). Here, 'optimal' refers to the conditions yielding the highest level of soluble active target protein, or, if no activity assay exists, simply the conditions yielding the most target protein (but see caveats below). Although the level of solubilized target protein usually increases with time, many membrane proteins show reduced stability in detergent. It is advantageous, and more common, to minimize solubilization times (*i.e.* <4 h), thereby facilitating a more rapid purification. Physical methods such as sonication and passage through a cell disruptor may also be used to speed the solubilization process. Target proteins are considered soluble if, in the presence of detergent, they remain in solution after being subjected to 100 000 *g* for 1 h.

3.2.3.4. General considerations for monitoring membrane-protein activity

Although often overlooked (surprisingly so), developing a means of following membrane-protein activity can prove critical when optimizing solubilization and purification conditions. Unfortunately, there is no universal method for assaying the activity of all solubilized or purified membrane proteins. For receptors, obvious assays include those based on monitoring ligand binding. For many other targets, such as those critically dependent on the vectorial nature of the membrane for activity (*i.e.* ion channels), there may be no direct assay in the detergent-solubilized state. On the premise that *any* assay is better than none, some simple biophysical analyses are worth mentioning. Firstly, some membrane targets that function as multimers have been shown to retain quaternary structure in sodium dodecyl sulfate (SDS) and during polyacrylamide gel electrophoresis (PAGE) (Cortes & Perozo, 1997; Prive, 2007). This observation can provide a convenient means of monitoring quaternary structure under various solubilization or purification conditions. It has also been suggested that proteins demonstrating this behaviour may, in general, be stable in a variety of detergents, as is the case for the KcsA potassium channel (Cortes & Perozo, 1997). Secondly, for those multimeric targets whose quaternary structure is not maintained in SDS, monitoring the degree of cross-linking in various detergents/conditions can provide for a simple, albeit indirect, structural probe (Sukharev *et al.*, 1999). Thirdly, monitoring the sensitivity of a target protein to limiting amounts of protease can also provide a simple means of probing 'activity' or structure under various solubilization/purification conditions. For example, binding to magnesium alters the protease sensitivity of detergent-solubilized CorA channels, and this can be easily monitored by SDS-PAGE analysis of digested samples (Payandeh & Pai, 2006). In theory, such an assay could be used to probe structural changes upon ligand binding for almost any target, so long as a ligand is known. The growing use of Fab antibody fragments as aids to membrane-protein crystallization provides a fourth assay for some targets (Hunte & Michel, 2002; Day *et al.*, 2007). Antibodies (or antibody fragments) that bind a target protein in a cellular environment should also be able to bind that protein in detergent, as evaluated by an assay such as immunoprecipitation. Ideally, such antibodies are

western-negative (*i.e.* they recognize nonlinear peptide epitopes that would only be present in a properly folded protein). Lastly, membrane proteins containing disulfide bonds (common in extracellular domains) often migrate more rapidly in SDS-PAGE than the same protein under reducing conditions. This can be used as an assay for proper protein folding in the cell, although this method does not provide information about target-protein stability in detergent.

3.2.3.5. Primary purification: affinity chromatography

Given that most membrane proteins are cloned with a histidine tag, the most common method for their primary purification is immobilized metal affinity chromatography (IMAC), used in >80% of recombinant membrane-protein structures (Willis & Koth, 2008; Newby *et al.*, 2009). IMAC methods for membrane proteins do not differ significantly from those commonly used for soluble proteins, with the notable exception that detergent is required in all buffers. During the wash and elution steps, the detergent concentration is typically reduced to a level above the CMC that is only just sufficient to maintain target-protein solubility. Ideally, the ratio of micelles to membrane-protein molecules is ~ 2 at this and subsequent purification stages [for an in-depth analysis, the reader is strongly encouraged to read Helenius *et al.* (1979) and Wiener (2004)]. The histidine tag may be removed after IMAC elution, although it is also worthwhile to proceed with a sample that retains the tag, since they provide critical lattice contacts in some protein crystals (Carson *et al.*, 2007).

3.2.3.6. Secondary purification: size-exclusion and ion-exchange chromatography

For most successful structures, target membrane proteins are sufficiently pure for crystal trials after a two-step purification: IMAC followed by size-exclusion chromatography (SEC) or ion-exchange chromatography (IEC) (Willis & Koth, 2008; Newby *et al.*, 2009). A detailed strategy is outlined in Fig. 3.2.3.1. SEC is the most common secondary (*i.e.* post-IMAC) method, represented in about half of all successful membrane-protein structures (Willis & Koth, 2008). IEC methods have been used less frequently, being employed for about one out of every four structures (Willis & Koth, 2008). The simple fact that some integral membrane proteins lack sufficient polar area to interact effectively with ion-exchange resins may account for the difference. Both methods permit the exchange of one detergent (*i.e.* that used for solubilization) for another or to a higher grade (a consideration, given the high cost of many detergents). Ion-exchange methods are likely to give a more complete detergent exchange, although this does not necessarily improve crystallization outcomes (Lemieux *et al.*, 2003). In fact, incomplete detergent exchange and/or incomplete removal of specifically bound lipids can prove beneficial in downstream crystal trials (Long *et al.*, 2007).

3.2.3.7. Concentrating membrane-protein samples for crystallization

Concentration of the target membrane protein is typically required prior to crystallization. Many different concentration methods can be used (*i.e.* those employed for soluble proteins), although some caution is warranted. Detergent will normally concentrate with the protein, since most detergent micelles are too large to pass through typical dialysis or concentration membranes. This can be detrimental to crystallization; high

detergent concentrations can denature protein or limit diffraction (Wiener, 2004). As a general rule, the highest-molecular-weight cutoff membrane that does not permit passage of the target protein should be used, as this will allow the passage of at least some protein-free detergent micelles. Also, any size-exclusion steps should be performed at a relatively high protein concentration (10–20 mg ml⁻¹ or higher) to minimize volumes. Purified protein may also be dialysed to exchange non-detergent buffer components, so long as the detergent concentration of the bulk solution is maintained just above the CMC. However, the dialysis of many detergents will proceed slowly (days to weeks). Accordingly, dialysis does not appear to be a widely used method for controlling detergent concentration prior to crystallization (reviewed by Willis & Koth, 2008).

3.2.4. Common purification pitfalls and prioritized alternative strategies

3.2.4.1. Poor solubility of the target protein

If the target protein does not remain soluble during purification, an obvious alternative strategy is to test different detergents or detergent combinations. Given that just a handful of different detergents have been used to solubilize and crystallize most membrane proteins, testing alternatives is usually not a daunting task. The most common method of detergent exchange is SEC (Fig. 3.2.3.1).

The zwitterionic lyso-lipid mimetic detergents such as FOSCHOLINE 12 (dodecylphosphatidylcholine) or 14 (FC12 and FC14; Anatrace, Maumee, Ohio, USA) will solubilize many membrane proteins (Eshaghi *et al.*, 2005) and there are a few reports of their use in crystallography [MscS mechanosensitive channel (Bass *et al.*, 2002); protein-conducting channel (van den Berg *et al.*, 2004)]. Should solubilization screening with the more commonly used detergents prove ineffective, it may be possible to solubilize first with a 'stronger' more lipid-like detergent, such as FC12, and then exchange to a non-charged detergent, such as DDM, during purification.

3.2.4.2. The isolated target protein does not crystallize

Although an in-depth analysis of membrane-protein crystallization methods is beyond the scope of this chapter (see instead Chapter 4.2), there are many purification parameters that can be varied to improve crystallization outcomes should initial trials fail to yield 'hits' or well diffracting crystals. Perhaps the foremost method is to exchange the protein into an alternative detergent or detergent mixture, typically by SEC, and repeat the crystal trials. Since the vast majority of membrane-protein structures have used one of only a handful of detergents (see above), it is likely that sampling even just a small number of other detergents would dramatically increase the likelihood of obtaining or optimizing crystals. Also, an increase in the alkyl-chain length by one methylene group (*i.e.* nonyl- versus octyl- β -D-glucoside) often leads to an increase in protein stability, and this too can improve crystallization outcomes (Wiener, 2004). If significant covalent disulfide aggregation of the protein is observed during purification, alkylation of free cysteines should be considered during the early stages of target-protein isolation. Other parameters that could be varied include the solution pH, salt concentration and temperature, or supplementing with known ligands, to name just a few.

3.2.5. Summary

There are many possible routes for the expression and isolation of membrane proteins for structural studies, and many factors can affect the likelihood of obtaining diffraction-quality crystals. The daunting prospect for almost any new membrane target is that every stage, including expression, solubilization, purification and crystallization, will prove challenging. In this light, it is encouraging that remarkably similar techniques have been employed for many successfully crystallized membrane targets, including, for example, the observation that most structures have been solved using just a handful of different detergents throughout the isolation and crystallization steps. It is equally significant that simple two-step purification strategies predominate. Clearly, new methods for the production of membrane proteins will continue to be developed. Nevertheless, for a field that is quite likely to be entering an acute phase of growth, a solid foundation has been established for reasonable 'first-try' strategies.

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