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High-throughput analytical gel filtration screening of integral membrane proteins for structural studies

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ABSTRACT

Background: Structural studies of integral membrane proteins (IMPs) are often hampered by difficulties in producing stable homogenous samples for crystallization. To overcome this hurdle it has become common practice to screen large numbers of target proteins to find suitable candidates for crystallization. For such an approach to be effective, an efficient screening strategy is imperative. To this end, strategies have been developed that involve the use of green fluorescent protein (GFP) fusion constructs. However, these approaches suffer from two drawbacks; proteins with a translocated C-terminus cannot be tested and scale-up from analytical to preparative purification is often non-trivial and may require re-cloning.
Methods: Here we present a screening approach that prioritizes IMP targets based on three criteria: expression level, detergent solubilization yield and homogeneity as determined by high-throughput small-scale immobilized metal affinity chromatography (IMAC) and automated size-exclusion chromatography (SEC).
Results: To validate the strategy, we screened 48 prokaryotic IMPs in two different vectors and two *Escherichia coli* strains. A set of 11 proteins passed all preset quality control checkpoints and was subjected to crystallization trials. Four of these crystallized directly in initial sparse matrix screens, highlighting the robustness of the strategy.
Conclusions: We have developed a rapid and cost efficient screening strategy that can be used for all IMPs regardless of topology. The analytical steps have been designed to be a good mimic of preparative purification, which greatly facilitates scale-up.
General significance: The screening approach presented here is intended and expected to help drive forward structural biology of membrane proteins.

1. Introduction

Integral membrane proteins (IMPs) perform a wide range of biological functions ranging from the transport of information, solutes and secondary metabolites between extracellular and intracellular milieus to the energy genesis in respiratory and photosynthetic complexes. Their genes constitute almost 30% of all open reading frames in the sequenced pro- and eukaryotic genomes [1]. The fact that about 60% of current therapeutics target membrane proteins, further underlines their significance [2–4]. However, in spite of their biological and pharmaceutical importance, structural data on IMPs are still limited. In fact, IMP structures comprise less than 2% of the entries in the Protein Data Bank (see www.pdb.org) [5]. This percentage is however likely to rise, as recent improvements on the level of gene and protein engineering, crystallization, microfocus X-ray diffraction, as well as NMR-methodology, have significantly increased the rate of IMP structure determinations in the last five years [6–13]. This has also inspired more scientists to take on the challenge of membrane protein structural biology. Nevertheless, tremendous

Abbreviations: IMP, integral membrane protein; GFP, green fluorescent protein; FSEC, fluorescence-detected size-exclusion chromatography; IMAC, immobilized metal affinity chromatography purification; SEC, size-exclusion chromatography; GF, gel filtration; AGF, analytical gel filtration; *E. coli*, *Escherichia coli*; LIC, ligation-independent cloning; TB, terrific broth; LB, Luria Bertani; IPTG, isopropyl-ß-D-thiogalactopyranoside; OD_{600nm}, optical density at 600 nm; HRP, horseradish peroxidase; DDM, dodecyl-β-D-maltoside; LDAO, N,N-dimethyldodecylamine-N-oxide; CYMAL-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; FC12, fos-choline-12; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEV, tobacco etch virus; GpA, glycophorin A; MATE, multidrug and toxic compound extrusion; BASS, bile acid sodium symporter; POT, proton dependent oligopeptide transporter; BisTris, bis(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)-methane; BSA, bovine serum albumin; TCEP, tris(2-carboxyethyl)phosphine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid

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efforts are still often necessary to obtain crystal structures of IMPs due to factors such as low expression levels, inefficient detergent extraction from the membrane, limited stability in detergent solution and poor crystal packing due to the presence of the micelle girdle as well as possible detrimental effects of free micelles [14,15]. Hence, finding suitable targets and determining optimal expression, purification and crystallization conditions generally requires extensive screening [11]. Fusing green fluorescent protein (GFP) to IMPs facilitates some steps in this screening process [16,17]. Here, the homogeneity of IMP–GFP fusion constructs can be assessed on the detergent solubilized crude cell lysates by fluorescence-detected size-exclusion chromatography (FSEC) [18,19]. This gives preliminary information on the quality of the over-expressed IMPs at an early time point in the process and requires little material. However, the GFP pipeline in bacteria is limited to membrane proteins with a cytoplasmic C-terminus, since GFP can only fold correctly and become fluorescent when it is localized in the cytoplasm [20]. A further drawback is that it is usually a requirement for downstream processes to cleave off the GFP moiety, which can be challenging due to masking effects of detergents on the protease recognition site [21–24]. Targets therefore often need to be re-cloned in GFP-tag-free vectors, requiring additional cloning and optimization steps [21]. Thus, although the screening of the homogeneity of the IMP in crude lysates using FSEC is a cheap and potentially time saving method, it is a relatively poor mimic of preparative purification.

Here we describe an alternative approach to rapidly screen and prioritize target proteins using high-throughput small-scale immobilized metal affinity chromatography (IMAC) followed by automated analytical SEC. This strategy greatly reduces time and costs for the optimal target selection and is amenable for all IMPs regardless of their topology. As the analytical steps have been designed to mimic preparative purification conditions, our approach ensures a high success rate in scale-up purification, which greatly accelerates the transition from analytical results to initial crystallization trials.

2. Materials and methods

2.1. Materials and reagents

All detergents were purchased from Affymetrix. Luria–Bertani, Miller (LB) and Terrific broth (TB) from Formedium. Kanamycin was obtained from Duchefa Biochemie and Isopropyl β-D-1-thiogalactopyranoside (IPTG) from Saveen Werner. All other chemicals were from Sigma-Aldrich, unless otherwise stated.

2.2. Construct design

Genes coding for the 48 different full-length IMPs were amplified from genomic DNA of the different organisms and cloned into the pNIC28-Bsa4 vector (N-terminal His-tag) and pNIC-CTHF (C-terminal His-tag) [25] using ligation independent cloning [26]. Both vectors possess a tobacco etch virus (TEV) cleavage site for Tag-removal. All vectors possess a T7 promoter and terminator sequence. The correct insertion of the gene sequence was verified by DNA sequencing.

2.3. SDS–PAGE and Western blot analysis

For gel electrophoresis, NuPAGE 4–12% Bis-Tris Gels (Life Technologies™) were used and stained by Coomassie Brilliant Blue R-250. Mark12 standard (Life Technologies™) or SeeBlue® Plus2 Prestained (Life Technologies™) were used as protein markers for SDS–PAGE and Western blots, respectively. Protein production levels were assessed by Western blotting of crude membranes (5 μl per lane; for details of crude membrane preparation see Section 2.4). As reference protein, crude membranes over expressing a C-terminal His-tagged (pNIC-CTHF-vector) version of the *Escherichia coli* peptide transporter ybgh, was used. Over-expression, purification and low resolution projection structure of this protein has recently been described [27] and yielded approximately 1 mg of purified protein per liter *of E. coli* culture. During Western blotting, proteins were transferred to nitrocellulose membranes using an iBLOT™ blotting system (Life Technologies™). Blots were blocked using 1% BSA in TBS-T buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.05% (v/v) Tween® 20) for 1 hour at room temperature. Membranes were washed 3 times for 10 min with TBS-T buffer and developed by incubation with a horseradish peroxidase-labeled His-probe (HisProbe™-HRP, Thermo Scientific) that recognizes poly-histidine tagged fusion proteins. Western blots were developed by Super Signal West Pico (Thermo Scientific) chemiluminescent substrate. Signals were detected and quantified using a Fluor-S™ MultiImager (Bio-Rad).

2.4. Small-scale protein expression and membrane preparation

Recombinant membrane proteins were over-expressed in *E. coli* BL21(DE3) and C41(DE3) cells. Cultures of 100 ml TB medium in 300 ml baffled conical flasks were inoculated from a LB overnight culture to a start OD_{600nm} of 0.05 per ml and grown at 37 °C at 200 rpm using an Infors shaker. At an OD_{600nm} of 0.7–1.0, the temperature was reduced to 20 °C over 60 min followed by IPTG induction (0.2 mM). Cultures continued to grow for further 16 hours prior to harvest. Cell density was monitored by measuring the OD_{600nm} value. Ninety milliliters of the cultures were harvested at 5,000 ×*g* for 6 min and the cell pellets were stored frozen at −80 °C.

Frozen cell pellets were thawed on ice and resuspended in lysis buffer (20 mM Tris pH 8.0, 300 mM NaCl, 1 mg/ml lysozyme, 5 U/ml DNase I, 100× diluted EDTA free protease inhibitor cocktail (Roche)) and lysed by using a high pressure homogenizer (Avestin C3) at 15,000 p.s.i. chamber pressure (three cycles). Crude membranes were harvested using ultracentrifugation at 104,000 ×*g* (Beckman Coulter Ti45 rotor) for 50 min. Membranes derived from 200 OD_{600nm} units were resuspended in 3 ml solubilization buffer (20 mM sodium phosphate buffer pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 5% glycerol and 100× diluted EDTA free protease inhibitor cocktail (Roche)). Aliquots were flash-frozen in liquid nitrogen and stored at −80 °C until further use.

2.5. Solubilization and small-scale purification

Based on Western blot results, membranes were chosen for small-scale IMAC purification followed by analytical gel filtration runs. For each target protein, 500 μl of membranes were solubilized in the presence of 1% of the four detergents (FC12, Cymal5, DDM, or LDAO) with stirring for 60 min at 4 °C. Non-solubilized material was removed by ultracentrifugation (104,000 ×*g* at 4 °C for 15 min).

Small-scale parallel affinity protein purification was performed using His MultiTrap FF (GE Healthcare) 96-well plates in the centrifugation modus (100 ×*g* for 1 min) according to the manufacturer's specification. In short, 500 μl of solubilized membranes where incubated for 45 min at 4 °C in batch in the His MultiTrap FF 96-well plate containing 50 μl of beads per well. Unspecifically bound proteins were removed by three washing steps with 200 μl of IMAC wash buffer (20 mM sodium phosphate buffer pH 7.5, 300 mM NaCl, 40 mM imidazole, 0.5 mM TCEP and 5% glycerol). Target proteins were eluted by addition of IMAC elution buffer (20 mM sodium phosphate buffer pH 7.5, 150 mM NaCl, 500 mM imidazole, 0.5 mM TCEP and 5% glycerol). All buffers contained the detergent used for solubilization with the following concentrations: 0.1% FC12, 0.03% DDM, 0.1% LDAO, 0.25% CYMAL-5. Fractions eluted with IMAC elution buffer were analyzed on Coomassie stained SDS–PAGE gels.

To assess the quality of the purified membrane protein, IMAC eluted samples were analyzed by gel filtration on a Superdex™ 200 5/150 GL analytical gel filtration column using an ÄKTAmicro™ chromatography system (GE Healthcare) equipped with the Autosampler A-905, which