Preparation of a Deuterated Membrane Protein for Small-Angle Neutron Scattering

Yuqi Wu[1], Kevin L. Weiss[2], and Raquel L. Lieberman[1]

Running header: Deuterated IAP for SANS

[1]School of Chemistry & Biochemistry, Georgia Institute of Technology

[2]Neutron Scattering Division, Oak Ridge National Laboratory

Abstract

This chapter outlines a protocol developed to prepare a purified deuterated membrane protein for a small-angle neutron scattering (SANS) experiment. SANS is a non-invasive technique well suited to studying membrane protein solution structures, and deuteration enhances the signal from the protein over the background [1,2]. We present our workflow: transformation of our plasmid into *E. coli*, cell growth and expression of our deuterated protein, membrane isolation, detergent solubilization, protein purification, purity assessment, and final preparation for SANS.

Keywords

Membrane protein, α-helix, recombinant expression, intramembrane proteolysis, detergent, contrast match point, deuteration, neutron scattering.

1 Introduction

Membrane proteins are abundant and important players in many fundamental cell processes, including catalysis, cell adhesion, and the transport of molecules across membranes. As such, about half of the current drugs target membrane proteins [3]. Still, relatively few molecular structures of membrane proteins are available (http://blanco.biomol.uci.edu/mpstruc/listAll/list), especially in comparison to structures of soluble proteins deposited in the protein data bank (http://www.rcsb.org/pdb). There are numerous challenges associated with the study of the functions and structures of membrane proteins [4,5]. Recombinant expression of human proteins is a common bottleneck, as the yield from a eukaryotic expression system is often low compared to levels needed for downstream applications for molecular characterization. Another problem is inherent in the nature of membrane proteins; detergent or other amphiphilic systems need to be used to maintain their active conformations [6]. Such reagents are not compatible with many downstream analytical techniques. For example, in crystallography the phase behavior of the surfactant can inhibit crystal growth [7], and in small-angle X-ray scattering (SAXS) [8] and in size exclusion chromatography coupled to multi-angle light scattering [9], the signal of the protein cannot always be readily distinguished from the signal from a similarly-sized detergent micelle.

Among the biophysical techniques currently available for studying membrane proteins, neutron scattering stands out [1,2]. Thermal or cold neutrons do not deposit energy into the samples, so unlike electrons or X-rays [10], neutrons are non-invasive and non-destructive to the sample [2]. Second, the method can be used to probe structure in lipid membranes or solution, as in small-angle neutron scattering (SANS). The signal from lipid or detergent can be suppressed using a method called “contrast matching”, which exploits the different scattering length densities (SLDs) of components such as proteins, detergents, and buffer, so that only the signal from the protein of interest remains [1,11]. Traditionally, the SLDs of solvent and detergent is matched by adjusting the percentage of D_2O [12], but

more sophisticated methods are emerging [13-16]. In addition, deuteration of the target macromolecule increases signal-to-noise, facilitating the data collection process [1,17,12].

Intramembrane aspartyl proteases (IAPs) are a subfamily of membrane-bound proteases that contain the conserved catalytic motif in YD…GXGD, where X is often a hydrophobic residue [18]. There are two main members of the IAP subfamily in humans: signal peptide peptidase (SPP) and presenilin. SPPs hydrolyze signal peptides and are involved, for example, in the production of the cell surface histocompatibility antigen–E epitope [19] and are exploited by hepatitis C for virus core protein maturation [20]. Presenilin is the catalytic subunit of the γ-secretase complex and is of interest due to its relevance to Alzheimer's disease [21,22]. Disease-causing mutations in presenilin or in one of its substrates, amyloid precursor protein (APP), alter the levels or atomic properties of amyloid-β, which ultimately deposit as plaques in the brain [23,21]. Human presenilin cannot be studied structurally in solution by SANS because it is only active after the full γ-secretase complex is assembled, and after it is endoproteolyzed into N-terminal and C-terminal fragments [21,22]. Human SPP and γ-secretase, which are expressed in eukaryotic cell lines, cannot be deuterated because eukaryotic cells cannot grow in media with more than 30-50% D_2O [24]. Therefore, to gain insight into the structural and biochemical properties of presenilin, we turned to an archaeal IAP ortholog from a methanogen *Methanoculleus marisnigri* JR1 (MCMJR1) as a model. MCMJR1 IAP (MCMIAP) and presenilin share sequence and structural similarity, including catalytic motifs within the same TM segments [25-27].

The protocol presented in this chapter describes the preparation of deuterated MCMIAP for SANS in order to study its structure and oligomeric state in detergent solution where it is enzymatically active [28,29]. The MCMIAP gene was cloned into a commercially available vector flanked by an N-terminal *pelB* leader sequence for periplasmic insertion and a C-terminal hexahistidine tag for affinity chromatography (Subheading 3.1) for expression and purification in relatively high yield [30]. After transforming chemically competent *E. coli* (Subheading 3.2) with the MCMIAP-containing plasmid, cells

were adapted to deuterium-labeled medium and then scaled up for overexpression (Subheading 3.3-3.7). Following cell lysis and membrane isolation (Subheading 3.7), purification consisted of detergent solubilization of the membranes, affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resin, and further polishing using size exclusion chromatography (SEC). SEC was also used to exchange the protein into a buffer solution with the desired D_2O and deuterated detergent content before analysis by SDS-PAGE (Subheading 3.8) and SANS. This protocol was developed in close collaboration with the Center for Structural Molecular Biology Bio-Deuteration Laboratory at Oak Ridge National Laboratory and should be adaptable to other membrane proteins with transmembrane (TM) helices or with β-barrels expressed in *E. coli*.

2 Materials

2.1 Molecular biology

1. The plasmid containing the gene of interest is the same as for the unlabeled protein [30].

2.2 Transformation of a suitable E. coli host

1. *E. coli* Rosetta™ 2(DE3) Singles™ competent cells (Novagen) (See Note 1).
2. Plasmid containing the gene of interest (see Subheading 3.1).
3. LB agar (e.g. LB Agar, Miller, Fisher Scientific) and petri dish.
4. Antibiotics for plasmid selection (ampicillin and chloramphenicol) (See Note 2).
5. SOC outgrowth medium (e.g. New England Biolabs).
6. Water bath set to 42 °C.
7. Incubator set to 37 °C.
8. Floor shaking incubator set to 37 °C.

2.3 Media and solutions

1. LB agar plate with single colonies of transformed *E. coli* cells (see Subheading 3.2).
2. LB broth (e.g. Difco™ LB Broth, Miller, BD).
3. Minimal media salts (Table 1).
4. D_2O (e.g. 99.8 atom % D deuterium oxide, Sigma-Aldrich).
5. Dry, sterile media bottles with GL45 threads.
6. Bottle top filters, 0.2 micron.
7. Appropriate antibiotics (e.g. carbenicillin and chloramphenicol) (See Note 2).
8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (e.g. Teknova).
9. Syringe or bottle filters, 0.2 micron.
10. 10% (w/v) NaOH in 90% D_2O (200 mL).
11. 30% (w/v) glycerol, 0.2% $MgSO_4 \cdot 7H_2O$ in 90% D_2O with antibiotics (300 mL).
12. 70% (v/v) ethanol.

2.4 Growth of deuterated inoculum

1. Shaking incubator.
2. Spectrophotometer (e.g. GE Ultrospec 10 cell density meter) for measuring optical density at 600 nm (OD_{600}) (See Note 3).
3. Baffled Fernbach shake flask.
4. Fixed angle centrifuge and rotor suitable for ~6,000xg at 4 °C.

2.5 Preparation of the bioreactor vessel and accessories

1. Benchtop bioreactor system (e.g. Eppendorf BioFlo 310).
2. Bioreactor vessel (e.g. Eppendorf).
3. Inlet tubing 3/8" ID x 1/2" OD x 1/16" wall (e.g. VWR).
4. Exhaust tubing 5/16" ID x 9/16" OD x 1/8" wall (e.g. Versilic).
5. Inlet vent filter, 0.2 micron (e.g. Pall Acro 37).

6. Exhaust vent filter, 0.2 micron (e.g. Pall Acro 50).
7. Autoclave.
8. Electrode, pH (e.g. Mettler Toledo).
9. Calibration buffers (pH 4 and pH 7).
10. Dissolved oxygen (DO) sensor (e.g. Mettler Toledo).
11. DO electrolyte solution.
12. House cooling water or recirculating chiller.
13. House compressed air and regulator with a gas purifier (W. A. Hammond Drierite).
14. Nitrogen tank and regulator.
15. Stainless steel headpieces (Sartorius Stedim) with gaskets (Sartorius Stedim).
16. Vent filter, 0.2 micron (e.g. GE Whatman 25mm Polyvent filter).
17. PharMed peristaltic tubing 1/16" ID x 3/16" OD x 1/16" wall (e.g. Saint-Gobain).
18. Polypropylene Glycol 2000 (Alfa Aesar).

2.6 Expression of deuterated membrane protein

1. Deuterated inoculum (see Subheading 3.4).
2. Spectrophotometer (e.g. GE Ultrospec 10 cell density meter) for measuring optical density at 600 nm (OD600) (See Note 3).
3. Semi-micro cuvettes, polystyrene.
4. 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution in 90% D_2O.

2.7 Membrane isolation from harvested cell paste

1. Cell paste containing the protein of interest (see Subheading 3.6).
2. Protease inhibitor (e.g. EDTA-Free SIGMAFAST Protease Inhibitor Cocktail Tablets, Sigma Aldrich).
3. Cell disruptor (e.g. Avestin EmulsiFlex C3) (See Note 4).

4. Ultracentrifuge and rotor suitable for ~150,000xg at 4°C.
5. Dounce homogenizer, 40 mL, with loose and tight pestles (See Note 5).
6. Cell resuspension buffer: 50 mM HEPES (pH 7.5) and 200 mM NaCl.
7. Liquid N_2.

2.8 Purification of deuterated membrane protein

1. Isolated membrane containing the protein of interest (see Subheading 3.7).
2. Dounce homogenizer, 40 mL, with loose and tight pestles (See Note 5).
3. Ni-NTA chromatography wash buffer (HisTrap buffer A): 50 mM HEPES (pH 7.5), 500 mM NaCl, and 20 mM imidazole.
4. Detergent for solubilizing membrane protein (e.g. Anagrade *n*-dodecyl β-D-maltoside (DDM), Anatrace), stored in desiccator at -80°C (See Note 6).
5. Ultracentrifuge and rotor suitable for 150,000xg at 4 °C.
6. Protein purification system (e.g. AKTA Pure). Depending on instrument, additional accessories may be required (e.g. Superloop 150 mL, GE Healthcare).
7. Ni-NTA column (e.g., HisTrap FF 1 or 5 mL, GE Healthcare) (See Note 7).
8. Wash buffer A for Ni-NTA chromatography: 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, and 0.1% DDM (See Note 8).
9. Elution buffer B for Ni-NTA chromatography: 50 mM HEPES (pH 7.5), 500 mM NaCl, 500 mM imidazole, and 0.1% DDM (See Note 8).
10. Gel filtration column (e.g. HiPrep 16/60 Sephacryl S-300, GE Healthcare).
11. Gel filtration buffer: 20 mM HEPES (pH 7.5), 250 mM NaCl, and 0.05% DDM (See Note 8).
12. Gel filtration column (e.g. Superose 12 10/300 GL, GE Healthcare).

13. Gel filtration buffer containing the correct D_2O percentage from contrast match point (CMP) calculation [11] (see Subheading 3.8.10) (See Note 9).
14. Amicon Ultra 15 mL centrifugal filter units (Millipore Sigma) with the appropriate molecular weight cut-off (MWCO) (See Note 10).
15. Swinging bucket benchtop centrifuge suited to ~4500xg at 4 °C.
16. Spectrophotometer for measuring protein absorbance at 280 nm.
17. 12% polyacrylamide gel.
18. Electrophoresis unit and power supply.
19. Imager (e.g. Amersham Imager 600).

3 Methods

3.1 Molecular biology

1. Clone the gene of the membrane protein of interest into pET-22b(+) vector (Novagen) using the procedure and considerations described previously [30] (Figure 1) (See Note 11).
2. Verify plasmid fidelity by DNA sequencing (e.g. MWG Operon or Genscript).

3.2 Transformation of a suitable E. coli host

1. Prepare an LB agar plate containing the appropriate amount of antibiotics (e.g., 60 µg/mL ampicillin and 34 µg/mL chloramphenicol) by following the manufacturer's protocol. Dry the plate overnight with lid slightly open at 37 ˚C in an incubator.
2. Thaw a ~50 µL aliquot of *E. coli* Rosetta 2 (DE3) cells on ice.
3. Add 30-50 ng plasmid DNA to the cells without mixing. Incubate the cells with DNA on ice for 30 min.

4. Heat shock the cells at 42 ˚C in the water bath for exactly 45 s. Return the cells to ice immediately afterwards.
5. After 2 min, add ~250 µL pre-warmed SOC outgrowth medium to the cells.
6. Shake the cells at 37 ˚C in a floor shaking incubator at 225 RPM for 1 hr.
7. Using sterile technique, spread ~250 µL cells on the pre-dried agar plate with a sterilized loop.
8. Incubate the agar plate at 37 ˚C in an incubator overnight.
9. The next morning, confirm the presence of well-dispersed single colonies on the transformation plate and continue with growth of the deuterated inoculum (Subheading 3.4) or seal the plate with Parafilm and store it at 4 ˚C for later use.

3.3 Preparation of media and solutions

1. Prepare LB broth in H_2O according to manufacturer's instructions.
2. Prepare minimal medium in H_2O and D_2O according to Table 1. To guard against precipitation, add the magnesium and trace metals solutions only after the other components have been dissolved and diluted to at least a 2X concentration.
3. Prepare 1 mL of a 100 mg/mL stock solution of ampicillin and carbenicillin in H_2O and D_2O. Prepare 1 mL of a 34 mg/mL stock solution of chloramphenicol in ethanol.
4. Prepare 5 mL of a 1M IPTG stock solution in H_2O and D_2O.
5. Using syringe or bottle top filters, filter all solutions into dry, autoclaved tubes or containers using sterile technique.

3.4 Growth of the deuterated inoculum

1. Using sterile technique, inoculate a single colony from the LB agar plate (see Subheading 3.2) into a clear polystyrene 17x100mm culture tube containing 3 mL LB broth and antibiotics.

2. Incubate the cells at 37 ˚C in a shaking incubator at 250 RPM.
3. When the OD_{600} of the cell culture reaches ~1, dilute the culture 1:20 (v/v) into 3 mL minimal medium (0% D_2O) in another polystyrene culture tube (See Note 12).
4. When the OD_{600} of the minimal medium culture reaches ~1, dilute the culture 1:20 (v/v) into 3 mL minimal medium containing 50% D_2O.
5. To adapt the cells to deuterium-labeled medium, repeat step 4 with minimal medium containing 75% and then 100% D_2O (or desired maximal percentage). To prepare fully deuterated (perdeuterated) protein, use 100% D_2O and a deuterated carbon source (e.g. glycerol-d8) (See Note 13).
6. Once the cells are growing in the deuterated medium that will be used for protein expression, progressively scale up the adapted culture in fresh deuterated medium to a final inoculum that is $1/10^{th}$ the initial volume to be used in the bioreactor. For the deuteration of MCMIAP, a 400 mL inoculum was prepared [14,11].

3.5 Preparation of the bioreactor vessel and accessories

1. Based on the protein expression level and the amount of material required, select a bioreactor vessel. For the deuteration of MCMIAP, a vessel with a maximum working volume of 5.5L was used [14,11].
2. Clean and assemble the bioreactor vessel per manufacturer's instructions. However, do not add the sensors (DO and pH) or deuterated medium.
3. Steam sterilize the empty bioreactor vessel, feed bottles, and peristaltic pump tubing in an autoclave.
4. Immediately after the autoclave cycle ends, carefully move the hot vessel to the bioreactor system and flow dry compressed air through the vessel via the inlet vent filter. Dry all other autoclaved labware in an oven set to 80 ˚C.

5. Prepare the DO sensor using manufacturer's instructions. Polarographic sensors must be polarized for several hours prior to calibration.
6. Attach the pH electrode to the bioreactor system and calibrate using pH standards.
7. Rinse the DO and pH sensors with deionized water and spray them down thoroughly with 70% ethanol.
8. After carefully blotting away the 70% ethanol, insert both sensors into the vessel and connect the corresponding cables. Insert the temperature probe into the thermowell within the vessel headplate.
9. Following manufacturer's instructions, connect the inlet and outlet water hoses to the corresponding ports on the vessel base and exhaust condenser.
10. Assemble two oven-dried feed bottles, add the filtered 10% (w/v) NaOH to one bottle and the 30% (w/v) glycerol feed solution to the other.
11. Attach peristaltic tubing to the bottles and pump heads then prime the lines with solution.
12. Pour sterile-filtered, deuterated medium into the vessel via an inoculation port.
13. Add 200 μl polypropylene glycol 2000 (antifoam) per liter of medium.
14. Install the motor assembly, agitate the medium, and enter the temperature setpoint.
15. Once the temperature stabilizes, calibrate the DO sensor with nitrogen and compressed air per manufacturer's instructions.
16. Enter the remaining setpoints at the bioreactor system console. See Table 2 for the setpoint values that were used for the deuteration of MCMIAP [14,11].
17. Enable cascaded control at the bioreactor system console to vary agitation (200-800 RPM) to maintain DO greater than 30%.

18. If available, initiate software for recording data and controlling the glycerol feed. The DO spike that occurs upon consumption of the initial 5 g/L of glycerol is typically used as the trigger that initiates glycerol feeding.

3.6 Expression of deuterated membrane protein

1. Inoculate the vessel with deuterium-labeled culture prepared in Subheading 3.4.
2. Monitor the bioreactor system and collect periodic samples from the vessel for OD_{600} measurements.
3. Once the deuterated cell mass increases (OD_{600} ~8-10), set the desired induction temperature and add IPTG. The induction time, temperature, and post-induction feed rate varies with the protein being expressed.
4. Harvest the cell paste by centrifugation at ~6,000×g for 45 min at 4 ˚C and shut down the bioreactor system following manufacturer's instructions.
5. Flash freeze the cell paste in liquid N_2 and store at -80 ˚C for later use or lyse directly as described in Subheading 3.7.

3.7 Membrane isolation from harvested cell paste

1. In centrifuge bottles or a separate glass media bottle, resuspend the deuterated cell paste in buffer (see Subheading 2.7.6) containing an appropriate amount of protease inhibitor on ice. For every 1 g of cell paste, use at least 5 mL of resuspension buffer. Resuspend the cell paste gently and thoroughly using a serological pipet.
2. Lyse the cells by three passages through a cell disruptor at 15,000 psi.
3. Centrifuge the cell lysate at ~5000×g for 15 min at 4 ˚C to remove cell debris. Repeat 3-4 times to obtain clear supernatant.
4. Continue with membrane isolation (step 5) or aliquot the supernatant in 50 mL Falcon tubes or Nalgene bottles and store at -80 ˚C.

5. Ultracentrifuge the supernatant at ~150,000×g for 30-45 min at 4 ˚C to isolate the membranes.
6. Pool the pelleted membranes into a 40 mL Dounce homogenizer. Resuspend the membrane in fresh cell resuspension buffer using 10-20 passes of the loose pestle and 3 passes of the tight pestle.
7. Ultracentrifuge again at 150,000×g for 30-45 min at 4 ˚C. Repeat steps 6 and 7 to completely remove loosely bound proteins.
8. Remove the pelleted membrane from the ultracentrifuge tube with a spatula. Use in purification immediately or flash freeze in liquid N_2 for storage at -80 ˚C for later use (See Note 14).

3.8 Purification of deuterated membrane protein

1. Add desired membrane mass to a 40 mL Dounce homogenizer using a spatula containing Ni-NTA wash buffer A (no DDM) on ice. For every 1 g of membrane, use 5 mL of buffer. If more than 40 mL is needed, divide the sample accordingly.
2. Resuspend the membrane fraction using 10-20 passes of the loose and tight pestles until membrane appears homogeneously resuspended in buffer.
3. After warming the DDM bottle to room temperature for 15-20 min, make a 2% DDM solution in Ni-NTA wash buffer A in a conical tube, matching the volume used for membrane resuspension. Place this solution on a 3D rocker for 10-15 min at 4 ˚C to fully dissolve the DDM.
4. Gently add the resuspended membranes from step 2 to the 2% DDM buffer solution from step 3; the final concentration of DDM will be 1%. Place the conical tube on the 3D rocker for 30-60 min at 4 ˚C to fully solubilize the membrane protein (Figure 2A) (See Note 15).

5. Ultracentrifuge at 150,000×g for 30-45 min at 4 ˚C to remove any insoluble material (Figure 2B).
6. Load the supernatant from step 5 (Figure 2C) onto the Ni-NTA column connected to a protein purification system and pre-equilibrated with Ni-NTA wash buffer A (containing 0.1% DDM). Wash with Ni-NTA wash buffer A until the absorbance returns to baseline, then wash with a mixture of 95% Ni-NTA wash buffer A and 5% Ni-NTA elution buffer B (containing 0.1% DDM) to remove unbound and loosely bound proteins. Elute the protein using a gradient from 5% to 60% Ni-NTA elution buffer B. Collect 1.5 mL fractions throughout the purification. For volumes higher than that of the Superloop, do consecutive purifications until all the supernatant has been processed (See Note 7).
7. As needed, concentrate each elution fraction to 500 µl using Amicon Ultra concentration devices centrifugal filter units with appropriate molecular weight cutoff (MWCO) (See Note 10). Analyze the fractions by SDS-PAGE to confirm that the protein of interest is present and contains minimal impurities (See Notes 7 and 16).
8. Pool elution fractions and further concentrate to 1 mL as in step 7 for polishing using SEC (e.g. HiPrep 16/60 Sephacryl S-300). Use gel filtration buffer (containing 0.05% DDM) for equilibration and elution.
9. To exchange purified protein into conditions for contrast matching in the SANS experiment, use the Superose 12 SEC column (Figure 3) (See Note 9).
10. After SDS-PAGE analysis to confirm purity (Figure 3), using concentration devices as needed, pool fractions and concentrate to the final volume for SANS (See Note 17).
11. Calculate the final concentration of the SANS sample by measuring the absorbance of the final sample at 280 nm and converting to concentration, for example, using the

calculated extinction coefficient (e.g. using online ProtParam tool [31]) and molecular weight of the protein (See Note 18).

Table 1. Materials for minimal media preparation [32,33].

Name of Salt		**Concentration**
$(NH_4)_2SO_4$		7.0 g/L
Na_2HPO_4		5.25 g/L
KH_2PO_4		1.6 g/L
Diammonium hydrogen citrate		0.50 g/L
Glycerol		5.0 g/L
20% (w/v) $MgSO_4 \cdot 7H_2O$		1.0 mL/L
Holme trace metals (1.0 mL/L)	$CaCl_2 \cdot H_2O$	0.50 g/L
	$CoCl_2$	0.098 g/L
	$CuSO_4$	0.102 g/L
	$FeCl_3 \cdot 6H_2O$	16.7 g/L
	$MnSO_4 \cdot H_2O$	0.114 g/L
	$Na_2EDTA \cdot 2H_2O$	22.3 g/L
	$ZnSO_4 \cdot H_2O$	0.112 g/L

Table 2. Initial Setpoints.

Setpoint Name	**Setpoint Value**

Temperature	30 ˚C
Dissolved Oxygen	30 %
pH	6.9
Agitation	200 RPM
Gas Flow	4 L/min
Glycerol Feed	~12 mL/hr

4 Notes

1. After optimization, *E. coli* Rosetta 2 (DE3) cells were selected to express MCMIAP because this line corrects for rare codons and active enzyme could be purified in sufficient yield for downstream applications. An option to avoid this cell line might be to conduct codon optimization on the plasmid [34].
2. Both antibiotics are used to select for only successfully transformed *E. coli* Rosetta 2 (DE3) cells ampicillin/carbenicillin resistance comes from the pET22b(+) plasmid (Figure 1). Chloramphenicol resistance comes from the plasmid which codes for rare codon tRNAs in *E. coli* Rosetta 2 (DE3) cells.
3. Since the measured OD_{600} is due to light scattering, which is affected by the optical layout, OD_{600} measurements made using different spectrophotometers can vary for the same culture [35].
4. Other cell disruption methods (sonication or French press) can be used depending on the scale required.
5. The Dounce homogenizer offers gentle homogenization and avoids disruption of the membrane.
6. The choice of detergent may vary for different proteins [5]. Previous detergent screening and protein stability tests in detergents suggested that DDM was optimal for the purification of MCMIAP [30].
7. It may be advantageous to use a larger (e.g. 5 mL or tandem 1 mL) HisTrap column for purification. In our experience, the column may not capture all of the protein of interest from the first round of column purification. Reloading the flow-through fractions onto a cleaned column may help rescue some of the protein of interest. Similarly, it is probably good practice to divide the samples into multiple batches and run each batch separately on a Ni-NTA column to prevent overloading the column.

8. 0.1% and 0.05% are much higher than the reported critical micelle concentration of DDM: 0.0087% [36]. Higher concentration ensures a high level of solubilization of membrane protein.
9. In our study of MCMIAP by SANS, 48.5% (w/v) tail-deuterated DDM (total with protonated DDM 0.05%) and 44% D_2O were used to extinguish the scattering signal from DDM [14]. A detailed protocol for determining the CMP can be found in Oliver *et al* [11].
10. For MCMIAP, use 50 kDa MWCO. To choose the optimal molecular mass cutoff for concentrating a membrane protein, both the protein loss and detergent loss need to be considered. For detailed procedures on choosing the best centrifugal filter, see other studies by us and others [30,37].
11. Important features include an N-terminal *pelB* leader sequence for periplasmic membrane insertion and a C-terminal hexahistidine tag for Ni-NTA purification flanking the gene of interest. Depending on the membrane protein topology, *pelB* may not be appropriate. Other slight plasmid variations for MCMIAP have been reported [38,39].
12. Dilutions other than 1:20 (v/v) may also be used. For small volume adaptation to media higher than 75% D_2O content, multiple dilutions are often grown in parallel to guard against failure.
13. Cell growth will slow as the D_2O content increases. The final D_2O percentage used in the deuteration of MCMIAP was 90% [14]. Due to the long growth times in minimal media, ampicillin is typically substituted with carbenicillin. For growth of the Rosetta 2(DE3) cells used in the expression of deuterated MCMIAP, the carbenicillin concentration used was 100 μg/mL. At 90% D_2O, the chloramphenicol concentration was decreased from 34 to 17 μg/mL [14].
14. Membranes can be stored at -80 ˚C for weeks. Start the purification close to the scheduled SANS beam time to ensure that the protein is as fresh as possible.

15. After rocking, the solution may turn from opaque to translucent, but if not, still proceed to ultracentrifugation step. An additional resuspension and solubilization round can be employed to ensure complete membrane solubilization.
16. For MCMIAP, the *E. coli* outer membrane protein (OmpF, MW~40 kDa monomer, ~100 kDa trimer) and multidrug efflux protein (AcrB, MW ~113 kDa monomer, ~300 kDa trimer) are common impurities identified during the purification process.
17. The final concentrated volume should consider the volume of quartz cells used in SANS experiment. Cylindrical quartz cells (banjo cells) with a 1mm pathlength require ~300 μL each. Besides SDS-PAGE, mass spectrometry methods can be used to confirm the identity of the protein, although the deuteration can complicate analysis for typical peptide digestion experiments.
18. Protein expression and purification procedures require optimization for each different membrane protein for optimal yield.

Acknowledgements

SANS studies on IAPS in the Lieberman lab are supported by NSF grant 1817796. The Office of Biological and Environmental Research supported research at Oak Ridge National Laboratory's Center for Structural Molecular Biology (CSMB), using facilities supported by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy.

References

1. Breyton C, Gabel F, Lethier M, Flayhan A, Durand G, Jault JM, Juillan-Binard C, Imbert L, Moulin M, Ravaud S, Hartlein M, Ebel C (2013) Small angle neutron scattering for the study of solubilised membrane proteins. Eur Phys J E Soft Matter 36 (7):71. doi:10.1140/epje/i2013-13071-6

2. Garg S, Porcar L, Woodka AC, Butler PD, Perez-Salas U (2011) Noninvasive neutron scattering measurements reveal slower cholesterol transport in model lipid membranes. Biophysical journal 101 (2):370-377. doi:10.1016/j.bpj.2011.06.014

3. Overington JP, Al-Lazikani B, Hopkins AL (2006) How many drug targets are there? Nat Rev Drug Discov 5 (12):993-996. doi:10.1038/nrd2199

4. Carpenter EP, Beis K, Cameron AD, Iwata S (2008) Overcoming the challenges of membrane protein crystallography. Curr Opin Struct Biol 18 (5):581-586. doi:10.1016/j.sbi.2008.07.001

5. Lin S-H, Guidotti G (2009) Chapter 35: Purification of membrane proteins. In: Burgess RR, Deutscher MP (eds) Methods in Enzymology, vol 463. Academic Press, pp 619-629. doi:https://doi.org/10.1016/S0076-6879(09)63035-4

6. Seddon AM, Curnow P, Booth PJ (2004) Membrane proteins, lipids and detergents: not just a soap opera. Biochim Biophys Acta 1666 (1-2):105-117. doi:10.1016/j.bbamem.2004.04.011

7. Wiener MC (2004) A pedestrian guide to membrane protein crystallization. Methods 34 (3):364-372. doi:https://doi.org/10.1016/j.ymeth.2004.03.025

8. Hong X, Weng YX, Li M (2004) Determination of the topological shape of integral membrane protein light-harvesting complex LH2 from photosynthetic bacteria in the detergent solution by small-angle X-ray scattering. Biophys J 86 (2):1082-1088. doi:10.1016/s0006-3495(04)74183-1

9. Gimpl K, Klement J, Keller S (2016) Characterising protein/detergent complexes by triple-detection size-exclusion chromatography. Biol Proced Online 18:4. doi:10.1186/s12575-015-0031-9

10. Shull CG, Wollan EO (1948) X-Ray, electron, and neutron diffraction. Science 108 (2795):69-75. doi:10.1126/science.108.2795.69

11. Oliver RC, Naing S-H, Weiss KL, Pingali SV, Lieberman RL, Urban VS (2018) Contrast-matching detergent in small-angle neutron scattering experiments for membrane protein structural analysis and ab Initio modeling. JoVE (140):e57901

12. Trewhella J (2006) Neutrons reveal how nature uses structural themes and variation in biological regulation. Physica B 385:825-830

13. Oliver RC, Pingali SV, Urban VS (2017) Designing mixed detergent micelles for uniform neutron contrast. J Phys Chem Lett 8 (20):5041-5046. doi:10.1021/acs.jpclett.7b02149

14. Naing SH, Oliver RC, Weiss KL, Urban VS, Lieberman RL (2018) Solution Structure of an Intramembrane Aspartyl Protease via Small Angle Neutron Scattering. Biophys J 114 (3):602-608. doi:10.1016/j.bpj.2017.12.017

15. Midtgaard SR, Darwish TA, Pedersen MC, Huda P, Larsen AH, Jensen GV, Kynde SAR, Skar-Gislinge N, Nielsen AJZ, Olesen C, Blaise M, Dorosz JJ, Thorsen TS, Venskutonyte R, Krintel C, Moller JV, Frielinghaus H, Gilbert EP, Martel A, Kastrup JS, Jensen PE, Nissen P, Arleth L (2018) Invisible detergents for structure determination of membrane proteins by small-angle neutron scattering. FEBS J 285 (2):357-371. doi:10.1111/febs.14345

16. Ashkar R, Bilheux HZ, Bordallo H, Briber R, Callaway DJE, Cheng X, Chu XQ, Curtis JE, Dadmun M, Fenimore P, Fushman D, Gabel F, Gupta K, Herberle F, Heinrich F, Hong L, Katsaras J, Kelman Z, Kharlampieva E, Kneller GR, Kovalevsky A, Krueger S, Langan P, Lieberman R, Liu Y, Losche M, Lyman E, Mao Y, Marino J, Mattos C, Meilleur F, Moody P, Nickels JD, O'Dell WB, O'Neill H, Perez-Salas U, Peters J, Petridis L, Sokolov AP, Stanley C, Wagner N, Weinrich M, Weiss K, Wymore T, Zhang Y, Smith JC (2018) Neutron scattering in the biological sciences: progress and prospects. Acta Crystallogr D Struct Biol 74 (Pt 12):1129-1168. doi:10.1107/S2059798318017503

17. Meilleur F, Weiss KL, Myles DA (2009) Deuterium labeling for neutron structure-function-dynamics analysis. Methods Mol Biol 544:281-292. doi:10.1007/978-1-59745-483-4_18

18. Erez E, Fass D, Bibi E (2009) How intramembrane proteases bury hydrolytic reactions in the membrane. Nature 459 (7245):371

19. Lemberg MK, Bland FA, Weihofen A, Braud VM, Martoglio B (2001) Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. J Immunol 167 (11):6441-6446. doi:10.4049/jimmunol.167.11.6441

20. McLauchlan J, Lemberg MK, Hope G, Martoglio B (2002) Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J 21 (15):3980-3988. doi:10.1093/emboj/cdf414

21. Wolfe MS (2019) Structure and Function of the gamma-Secretase Complex. Biochemistry 58 (27):2953-2966. doi:10.1021/acs.biochem.9b00401

22. Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398 (6727):513-517. doi:10.1038/19077

23. Chow VW, Mattson MP, Wong PC, Gleichmann M (2010) An overview of APP processing enzymes and products. Neuromolecular Med 12 (1):1-12. doi:10.1007/s12017-009-8104-z

24. Katz JJ, Crespi HL (1966) Deuterated organisms: cultivation and uses. Science 151 (3715):1187-1194. doi:10.1126/science.151.3715.1187

25. Dang S, Wu S, Wang J, Li H, Huang M, He W, Li YM, Wong CC, Shi Y (2015) Cleavage of amyloid precursor protein by an archaeal presenilin homologue PSH. Proc Natl Acad Sci U S A 112 (11):3344-3349. doi:10.1073/pnas.1502150112

26. Bai XC, Yan C, Yang G, Lu P, Ma D, Sun L, Zhou R, Scheres SHW, Shi Y (2015) An atomic structure of human gamma-secretase. Nature 525 (7568):212-217. doi:10.1038/nature14892

27. Li X, Dang S, Yan C, Gong X, Wang J, Shi Y (2013) Structure of a presenilin family intramembrane aspartate protease. Nature 493 (7430):56-61. doi:10.1038/nature11801

28. Naing SH, Kalyoncu S, Smalley DM, Kim H, Tao X, George JB, Jonke AP, Oliver RC, Urban VS, Torres MP, Lieberman RL (2018) Both positional and chemical variables control in vitro proteolytic cleavage of a presenilin ortholog. J Biol Chem 293 (13):4653-4663. doi:10.1074/jbc.RA117.001436

29. Naing SH, Vukoti KM, Drury JE, Johnson JL, Kalyoncu S, Hill SE, Torres MP, Lieberman RL (2015) Catalytic Properties of Intramembrane Aspartyl Protease Substrate Hydrolysis Evaluated Using a FRET Peptide Cleavage Assay. ACS Chem Biol 10 (9):2166-2174. doi:10.1021/acschembio.5b00305

30. Johnson JL, Kalyoncu S, Lieberman RL (2016) Lessons from an alpha-helical membrane enzyme: Expression, purification, and detergent optimization for biophysical and structural characterization. Methods Mol Biol 1432:281-301. doi:10.1007/978-1-4939-3637-3_18

31. Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF (1999) Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 112:531-552. doi:10.1385/1-59259-584-7:531

32. Holme T (1970) Enzymes-laboratory scale production. Process Biochem 5:62-66

33. Törnkvist M, Larsson G, Enfors S-O (1996) Protein release and foaming in Escherichia coli cultures grown in minimal medium. Bioprocess Eng 15 (5):231-237. doi:10.1007/bf02391583

34. Norholm MH, Light S, Virkki MT, Elofsson A, von Heijne G, Daley DO (2012) Manipulating the genetic code for membrane protein production: what have we learnt so far? Biochim Biophys Acta 1818 (4):1091-1096. doi:10.1016/j.bbamem.2011.08.018

35. Koch AL (1970) Turbidity measurements of bacterial cultures in some available commercial instruments. Anal Biochem 38 (1):252-259. doi:10.1016/0003-2697(70)90174-0

36. VanAken T, Foxall-VanAken S, Castleman S, Ferguson-Miller S (1986) Alkyl glycoside detergents: synthesis and applications to the study of membrane proteins. Methods Enzymol 125:27-35. doi:10.1016/s0076-6879(86)25005-3

37. Feroz H, Vandervelden C, Ikwuagwu B, Ferlez B, Baker CS, Lugar DJ, Grzelakowski M, Golbeck JH, Zydney AL, Kumar M (2016) Concentrating membrane proteins using ultrafiltration without concentrating detergents. Biotechnol Bioeng 113 (10):2122-2130. doi:10.1002/bit.25973

38. Zhou R, Shi Y, Yang G (2017) Expression, purification, and enzymatic characterization of intramembrane proteases. Methods Enzymol 584:127-155. doi:10.1016/bs.mie.2016.09.046

39. Torres-Arancivia C, Ross CM, Chavez J, Assur Z, Dolios G, Mancia F, Ubarretxena-Belandia I (2010) Identification of an archaeal presenilin-like intramembrane protease. PLoS One 5 (9). doi:10.1371/journal.pone.0013072

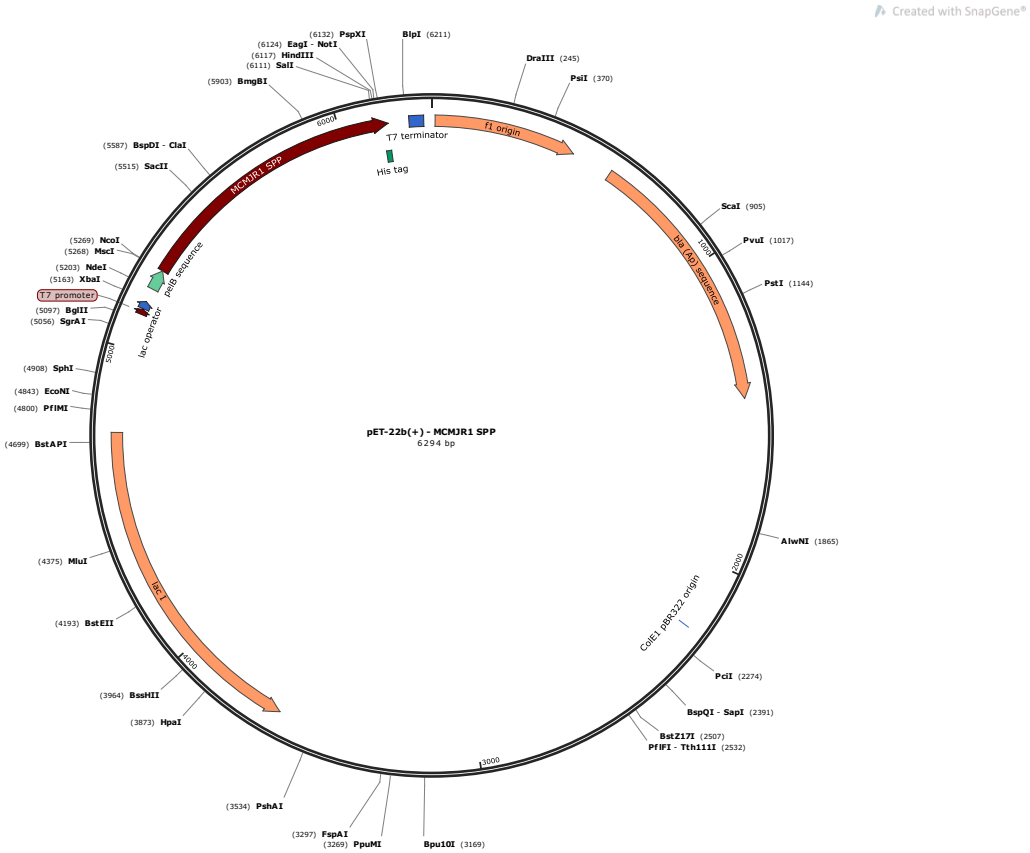

Figure 1. Plasmid map of pET-22b(+) cloned with MCMIAP (MCMJR1 SPP). The *pelB* sequence precedes the protein of interest, MCMIAP in this case, to be inserted into the periplasmic membrane. The hexahistidine tag enables purification through Ni-NTA chromatography. The bla (Ap) sequence codes for beta-lactamase, providing ampicillin resistance for the plasmid, which is used as a selection marker in the protocol. Figure was produced using SnapGene Viewer.

A **B** **C**

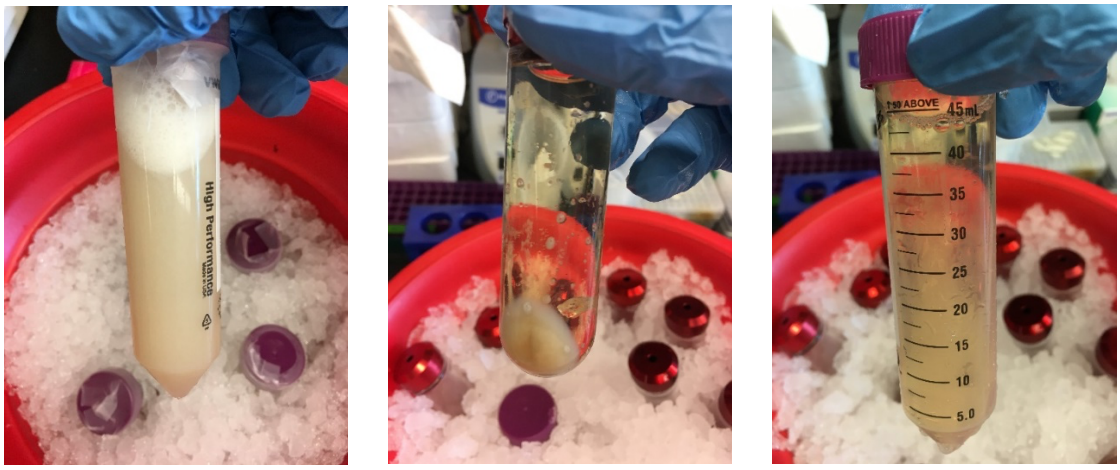

Figure 2. Membrane solubilization. (A) MCMIAP resuspended membranes with 1% DDM solution added. Note that the solution has not become translucent after rocking even though membranes are predominantly solubilized as seen in B. (B) Insoluble material is pelleted after ultracentrifugation, fully separated from the solubilized membrane protein, which is in solution. (C) Solubilized membrane fraction prior to Ni-NTA chromatography.

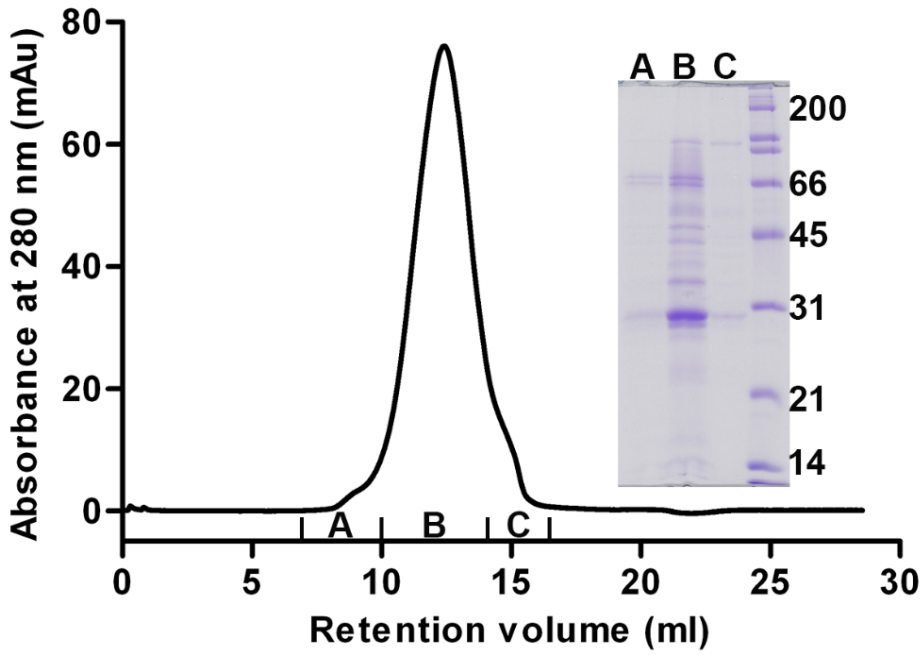

Figure 3. Purification of deuterated MCMIAP for SANS. Superose 12 column for final exchange into desired buffer (20 mM HEPES at pH 7.5, 250 mM NaCl, 48.5% D2O, 0.05% DDM, 44% (w/v) of which is tail-deuterated. The SDS-PAGE gel shows relatively clean MCMIAP (~31 kDa) in fraction B, which was used for SANS experiment. Figure is reproduced with permission from Naing et al [14].