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Isotopic labeling of eukaryotic membrane proteins for NMR studies of interactions and dynamics

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Abstract

Membrane proteins, and especially G-protein coupled receptors (GPCRs), are increasingly important targets of structural biology studies due to their involvement in many biomedically-critical pathways in humans. These proteins are often highly dynamic and thus benefit from studies by NMR spectroscopy in parallel with complementary crystallographic and cryo-EM analyses. However, such studies are often complicated by a range of practical concerns, including challenges in preparing suitably isotopically-labeled membrane protein samples, large sizes of protein/detergent or protein/lipid complexes, and limitations on sample concentrations and stabilities. Here we describe our approach to addressing these challenges via the use of simple eukaryotic expression systems and modified NMR experiments, using the human adenosine A_{2A} receptor as an example. Protocols are provided for the preparation of U-²H (¹³C, ¹H-Ile δ1) labeled membrane proteins from overexpression in the methylotrophic yeast *Pichia pastoris*, as well as techniques for studying the fast psec-nsec sidechain dynamics of the methyl groups of such samples. We believe that, with the proper optimization, these protocols should be generalizable to other GPCRs and human membrane proteins.

Keywords

NMR spectroscopy; membrane proteins; dynamics; relaxation; *Pichia pastoris*; isotopic labeling; GPCRs

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Introduction

Integral membrane proteins make up a large proportion of the genomes of many organisms – approximately 25% of the human genome – and perform a diverse range of functions, including key steps in the communication of a cell with its environment. Because of their biological and therapeutic importance (Almén, Nordström, Fredriksson, & Schiöth, 2009), membrane proteins are the focus of fundamental and applied biophysical research to characterize three-dimensional structures, dynamics, and interactions in native-like environments.

Solution state NMR spectroscopy has played a critical role in membrane protein biophysical studies, as the site-specific dynamic and interaction information provided by such approaches nicely complements structural data obtained from X-ray diffraction, cryo-EM and computational analyses (Cuniasse, Tavares, Orlova, & Zinn-Justin, 2017; Opella & Marassi, 2017). Fundamental to such studies are several 2D “fingerprint spectra,” most often $^{15}\text{N}/^1\text{H}$ HSQC (heteronuclear single quantum coherence) spectra (for backbone amide plus Trp, Asn and Gln sidechains) or methyl $^{13}\text{C}/^1\text{H}$ HMQC (heteronuclear multiple quantum coherence) spectra for sidechain methyl groups (Pellecchia, Bertini, Cowburn, Dalvit, Giralt, Jahnke et al., 2008). These methyl-directed experiments are especially advantageous for large, slow-tumbling membrane protein/lipid complexes; experiments directed to other sidechain and mainchain sites have been successfully applied as well. NMR experiments can provide information about protein dynamics over many time scales, from fast (psec-nsec) sidechain motions to slow conformational changes (μsec -msec) (Kasinath, Sharp, & Wand, 2013; Liang & Tamm, 2016; Palmer, 2012; Wand, Moorman, & Harpole, 2013). Many of these dynamics experiments, often using sidechain methyl groups as probes, have been adapted and developed for large biomolecular systems and can be used for membrane proteins (Rosenzweig & Kay, 2014; Sun, Kay, & Tugarinov, 2011; Tugarinov, Hwang, Ollerenshaw, & Kay, 2003).

A particular advantage of solution state NMR is that proteins are studied in a native-like solution state where they can interconvert among multiple conformations. However, membrane proteins must be solubilized in a suitable membrane mimetic that maintains native structure and dynamics. Different options include detergent micelles, amphipols, bicelles, nanodiscs, SMALPs, and lipid vesicles, each having their own benefits and drawbacks (Liang & Tamm, 2016, 2018; Zhou & Cross, 2013). It is often necessary to test different solubilization strategies for a given protein sample for stability, signal intensity and resolution, and native structure/activity. Two important considerations for all membrane mimetics are 1) a uniform and small particle size and 2) a high extent of deuteration.

High-level deuteration, both within the membrane mimetic and protein itself, is critical to reduce the number of ^1H signals present in spectra (including those from lipids, which can be intense) and to improve the relaxation characteristics of the remaining NMR-active spins in the sample. While deuteration is possible for the membrane-mimetic through the purchase / synthesis of deuterated compounds, replacing ^1H with ^2H in proteins requires biosynthetic incorporation. For backbone experiments in eukaryotic expression systems, one can label uniformly with ^{15}N to observe all amides (Eddy, Lee, Gao, White, Didenko, Horst

et al., 2018; Opitz, Isogai, & Grzesiek, 2015) or through addition of specifically-labeled amino acids (Isogai, Deupi, Opitz, Heydenreich, Tsai, Brueckner et al., 2016). For methyl groups, one can provide either appropriately-labeled amino acids or amino acid precursors (particularly alpha-keto acids) to growth media to access various labeling patterns in the sidechains of several amino acids (Kofuku, Ueda, Okude, Shiraishi, Kondo, Mizumura et al., 2014; Kofuku, Yokomizo, Imai, Shiraishi, Natsume, Itoh et al., 2018). We find isoleucine $\delta 1$ methyl groups particularly useful given 1) the abundance of Ile residues in integral membrane proteins including GPCRs (Ulmschneider & Sansom, 2001), 2) the far upfield ^{13}C shift of isoleucine $\delta 1$ methyl groups [average 13.5 ± 3.6 ppm ^{13}C according to BioMagResBank (Ulrich, Akutsu, Doreleijers, Harano, Ioannidis, Lin et al., 2008)], putting them in a particularly uncrowded region of 2D $^{13}\text{C}/^1\text{H}$ spectra, 3) the lack of need to stereospecifically assign these methyl groups, unlike Val and Leu, and 4) the presence of multiple, freely-rotatable bonds between the methyl group and protein backbone, providing substantial independence of dynamics at these sites (Kasinath et al., 2013).

While many of the aforementioned labeling strategies have been well developed for *E. coli*, many integral membrane proteins can only be expressed at high levels in eukaryotic hosts. Among these, the methylotrophic yeast *Pichia pastoris* is a convenient host for heterologous expression and isotopic labeling of eukaryotic membrane proteins (Clark, Dikiy, Rosenbaum, & Gardner, 2018). Advantages of *Pichia* include rapidity of genetic manipulation, high yields of recombinant protein, existence of post-translational modification and chaperone machinery necessary for eukaryotic membrane proteins, and ability to grow on defined minimal media allowing for perdeuteration (J. L. Cereghino & Cregg, 2000; Morgan, Kragt, & Feeney, 2000). Uniform isotopic labeling in *Pichia* has been well established (Morgan et al., 2000; Pickford & O'Leary, 2004). We have extended this work by demonstrating the ^{13}C , ^1H labeling of isoleucine $\delta 1$ -methyl groups in a perdeuterated background by adding labeled α -ketobutyrate (~50% labeling, ~90% deuteration) to highly deuterated growth media (Clark, Dikiy, Chapman, Rodstrom, Aramini, LeVine et al., 2017; Clark, Zahm, Ali, Kukula, Bian, Patrie et al., 2015). In contrast, simultaneous labeling of leucine δ - and valine γ -methyl groups with α -ketoisovalerate is inefficient but can be achieved by adding labeled valine directly to the growth media or modifying culture conditions (Clark et al., 2015; Suzuki, Sakakura, Mori, Fujii, Akashi, & Takahashi, 2018; Zhang, Yu, Xu, Jouhten, Swapna, Glaser et al., 2017). *Pichia* can readily take up additional amino acids from media, with a general correlation between uptake efficiency and the energetic cost to synthesize that amino acid type *de novo* (Heyland, Fu, Blank, & Schmid, 2011). However, after uptake into cells, labeled amino acids can be fed into metabolic pathways (Solà, Maaheimo, Ylonen, Ferrer, & Szyperski, 2004), diluting signal of desired amino acids and complicating data analysis by isotopic scrambling. Alternatively, auxotrophic strains can be developed for labeling a specific amino acid; however, care must be taken to confirm that off-target effects in other metabolic pathways do not arise (Whittaker, 2007).

Here we provide detailed protocols needed to generate such U- ^2H (^{13}C , ^1H -Ile $\delta 1$ methyl) labeled integral membrane proteins by overexpression in *Pichia*, using the human adenosine A_{2A} receptor [A_{2A}R] as a model system. We further detail how such samples can be used in solution NMR studies, from acquiring simple $^{13}\text{C}/^1\text{H}$ HMQC spectra, through chemical shift

assignments by site-directed mutagenesis, to analyses of ^1H - ^1H cross-relaxation measurements of fast sidechain dynamics.

Expression and isotopic labeling in *Pichia*

The overall strategy for expression of membrane proteins in *Pichia* is outlined below. After cloning the gene of interest into a *Pichia* expression vector [see Note 1 below and several literature reviews where such reagents are comprehensively discussed al.(Byrne, 2015; Cos, Ramon, Montesinos, & Valero, 2006; Fan, Emami, Munro, Ladizhansky, & Brown, 2015)], the total process to obtaining initial expression cultures takes about a week. While *Pichia* can be grown in large-scale fermenters (G. P. Cereghino, Cereghino, Ilgen, & Cregg, 2002), this protocol describes growth in shake-flasks, as this is the most straightforward and cost-efficient way to initiate *Pichia* expression at levels sufficient for structural biology work. Although the process outlined here is a good general starting point for membrane protein expression, it is likely that each sample will require optimization to achieve high levels of expression, and pertinent notes are indicated after each step.

Equipment

1. Plate incubator (28° C)
2. DNA agarose gel apparatus
3. Electroporator (e.g. Bio-Rad Micropulser) and cuvettes
4. Temperature-controlled shake flask incubator, ideally dedicated for *Pichia* expression

Required buffers and media (all solutions 0.2 μm sterile-filtered, store all at 4° C)

1. 1 M sorbitol
2. 20% w/v dextrose (use as 10x stock)
3. 10x YNB (per 1L: 34 g yeast nitrogen base without amino acids or nitrogen, 100 g ammonium sulfate)
4. 1 M Potassium phosphate pH 6.0 (use as 10x stock)
5. 2000x biotin (0.08% in 0.05 M NaOH)
6. 200x histidine (0.8% in water)
7. MD agar (1.5% agar, 1x YNB, 1x dextrose, 1x biotin, 1x histidine if required (“MDH” plates); add all stock solutions after autoclaving agar and water)
8. 10% w/v glycerol (use as 10 x stock)
9. YPD (1% yeast extract, 2% peptone, 1 x dextrose; dextrose solution added after autoclaving other components)
10. YPDS (YPD + 2% agar + 1 M sorbitol; sorbitol added after autoclaving)

11. BMG (1x glycerol, 1x potassium phosphate pH 6.0, 1x YNB, 1x biotin, and 1x histidine if required)
12. BMM without methanol (1x potassium phosphate pH 6.0, 1x YNB, 1x biotin, 1x histidine if required)

Procedure

1. Clone the gene of interest into the desired vector for expression in *Pichia*. The cDNA for wild-type human ADORA2A adenosine A_{2A} receptor with C-terminal His₆ and Protein C affinity tags was cloned into the pPICZ vector containing a modified MFα secretion signal (see **Note 1** below). Molecular biology steps were carried out using standard techniques and utilizing Zeocin selection.
 - a. **Note 1:** Although some membrane proteins have been successfully purified when expressing intracellularly (Byrne, 2015; J. Y. Lee, Kinch, Borek, Wang, Wang, Urbatsch et al., 2016), we have observed that the best expression of A_{2A}R occurred when the gene was preceded by the MFα signal sequence from *Saccharomyces cerevisiae*. Furthermore, the highest amount of fully-processed (e.g. plasma membrane localized) receptor occurred when the signal sequence was modified for more efficient processing. These modifications were based on previous literature precedents (Lin-Cereghino, Stark, Kim, Chang, Shaheen, Poerwanto et al., 2013; Rakestraw, Sazinsky, Piatetsi, Antipov, & Wittrup, 2009), comprising the V22A, G40D, L42S, V50A, V52A, and F55L mutations plus the deletion of residues 57–70 (Fig. 1).
 - b. **Note 2:** GPCRs and other membrane proteins commonly contain post-translational modifications (PTMs) and/or large flexible regions that are problematic for NMR or other structural analyses (Yurugi-Kobayashi, Asada, Shiroishi, Shimamura, Funamoto, Katsuta et al., 2009). PTM target residues can be removed through point mutations and flexible regions can be truncated during this stage. While *Pichia* does not hyperglycosylate overexpressed proteins to the extent of other yeast such as *S. cerevisiae* (Pickford & O’Leary, 2004), a simple way to minimize this possibility is to remove targeted asparagine residues. To avoid such issues with A_{2A}R, we mutated an N-glycosylation site at Asn154 to glutamine and truncated the construct at residue 316 as previously described (Jaakola, Griffith, Hanson, Cherezov, Chien, Lane et al., 2008).
2. Prepare a fresh batch of competent KM71H cells. Briefly, cells should be grown from a freshly streaked MD plate in YPD to an OD₆₀₀ of 1, then spun down under sterile conditions at 4000 rcf for 10 minutes. Cells were washed and repelleted in ice-cold, sterile Milli-Q water twice, followed by a wash in ice-cold 1 M sorbitol and resuspension in a minimal volume of 1 M sorbitol to create a thick, but pipettable, cell paste (e.g. ~500 μL for 500 mL pellet). Competent cells may be kept on ice at this point until electroporation.

- a. Note 1:** It is important that the competent cells are grown and prepared fresh for efficient transformation, preferably no more than 24 hours prior to electroporation. Sterility must be maintained as much as possible.
 - b. Note 2:** The popularity of *Pichia* as an expression host has expanded the options for expression and culturing conditions. *Pichia* is commercially available in various cell strains from several vendors including Invitrogen, including the most commonly used strains for MP expression (KM71/H, GS115, SMD1163/8, and wild-type X-33). KM71H is the strain that gave us the highest expression levels of A_{2A}R, although other membrane proteins have been successfully overexpressed and purified from alternative strains for structural studies (Byrne, 2015).
3. Purify 10–20 µg of plasmid prior to linearization. Linearize with *PmeI* or appropriate restriction enzyme and use a small amount of plasmid to verify efficient digestion with a DNA agarose gel. Re-purify linearized plasmid with PCR Clean-up Kit (Qiagen), with the final elution step carried out in a minimal volume of sterile Milli-Q water.
 - a. Note:** It is important to use a large amount of linearized plasmid (5 µg is ideal) for each electroporation to maximize efficiency of incorporation into the *Pichia* genome. If screening multiple *Pichia* strains, it is helpful to increase the scale of plasmid linearization and divide the resultant DNA between strains.
4. Add linearized plasmid to ~100 µL of competent *Pichia*, and transfer to an electroporation cuvette with a 0.2 cm gap. Include an extra cuvette containing cells and an equal volume of sterile water instead of plasmid as a negative control. Process each cuvette with 1 pulse of 2.00 kV (“Pic” program on Bio-Rad MicroPulser system), and immediately transfer cuvette back to ice. Add 1 mL of ice-cold 1 M sorbitol and transfer the contents of the cuvette to a 14 mL round-bottom culture tube.
 - a. Note:** Arcing during electroporation is rare but can happen if there is salt in the sample. This risk can be minimized by ensuring that the paste of competent cells has been thoroughly rinsed of media and eluting linearized plasmid with water instead of elution buffer.
5. Incubate cells at 28 °C for 30–60 minutes without shaking, and then add 1 mL YPD to each tube and shake at 200 rpm for 4 hours at 28 °C.
6. Plate cells on YPDS plates containing increasing amounts of Zeocin (100 µg/mL – 1000+ µg/mL) to check for efficiency of gene integration. Incubate plates at 28 °C for 3–4 days. “Pinprick” colonies should appear after 48 hours and individual colonies should be obvious (~1 mm diameter) after 72 hours.

7. Individual colonies should be expanded and screened for expression levels, generally through western blots, as simply using the copy number as a proxy for expression levels is not advisable (Aw & Polizzi, 2013). Typically, 10–12 clones from each sample are selected mostly (but not solely) from the plates containing the higher levels of Zeocin and easily expanded on a single MD (or MDH) plate. Individually pick colonies with a sterile pipet tip and gently spread each colony on a labeled area of the plate. After 24 hours of incubation at 28 °C, each colony will grow into a small “patch” that can be easily looped for screening cultures and subsequent preservation (see **Step 10** below).
8. Grow a small culture (~10 mL) of each clone by inoculating a loopful of yeast into sterile BMG media and incubating at 200 rpm/28 °C. After growing to saturation (OD_{600} ~20–30), spin down cells at 4000 rcf for 5 minutes. While maintaining sterility, remove media and replace with an equal volume of BMM media without methanol. After continued shaking at 28 °C for 4–6 hours to allow for metabolism of residual glycerol, induce expression with the addition of 0.5% methanol. Additional aliquots of 0.5% methanol should be added every 12 hours to maintain expression, and cells can be harvested and subjected to western blots after 48 hours.
 - a. **Note 1:** Although each sample will likely require individual optimization to achieve the best expression conditions, the strategy outlined above can be viewed as a general starting point. Common additives to increase expression levels are DMSO (André, Cherouati, Prual, Steffan, Zeder-Lutz, Magnin et al., 2006), histidine (even if not required by cell strain as it is an energetically “expensive” amino acid to synthesize; (Heyland et al., 2011)), and individual ligands, especially in the case of GPCRs (André et al., 2006; Clark et al., 2017; Hino, Arakawa, Iwanari, Yurugi-Kobayashi, Ikeda-Suno, Nakada-Nakura et al., 2012; Shimamura, Shiroishi, Weyand, Tsujimoto, Winter, Katritch et al., 2011). It is also common to use unbuffered media (J. Y. Lee et al., 2016), BMG/BMM at different pH levels (known to influence expression and activity levels of various GPCRs (André et al., 2006; Yurugi-Kobayashi et al., 2009)), or alter the temperature and length of expression (André et al., 2006; Yurugi-Kobayashi et al., 2009). In all cases, effects on protein expression can be assayed for with western blots.
 - b. **Note 2:** If methanol is not suitable for expression induction due to toxicity or other sample constraints, proteins can be expressed constitutively through use of the commercially available pGAPZ vectors (Invitrogen).
9. Once the best expression clone is identified, it can be stored as a glycerol stock at –80 °C and streaked onto fresh MD (or MDH) plates as needed. To prepare a culture for a glycerol stock, inoculate a loopful of the colony “patch” in YPD and

shake for 24 hours at 28 °C. Store culture in cryo vials with a final glycerol concentration of 25%.

10. To grow deuterated and isotopically labeled cultures for NMR experiments, use a loopful of freshly streaked cells from an MD (or MDH) plate and inoculate 50 mL of BMG media made with 90% D₂O / 10% H₂O. Shake the culture at 28 °C until it reaches an OD₆₀₀ of ~10 (approximately 36–48 hours). Use 100 µL of this culture to inoculate 50 mL of BMG media made with 100% D₂O. Shake the culture at 28 °C until it reaches an OD₆₀₀ of ~10 (approximately 36–48 hours). Use 100 µL of this culture to inoculate 50 mL of BMG media made with 100% D₂O, but replacing the 1% glycerol with 1% d₈-glycerol. Shake the culture until reaching an OD₆₀₀ of ~10, and then use the culture in its entirety to inoculate large-scale (1–2 L) cultures of identical media. Shake the large-scale cultures at 28 °C until reaching saturation (OD₆₀₀ of ~20–30), and then pellet cells in sterile 1 L bottles at 4000 rcf for 30 minutes. Remove the media and gently resuspend cell pellets in BMM media without methanol. Continue to shake flasks at 28 °C for ~12 more hours to metabolize any remaining d₈-glycerol. One hour prior to induction, add 200 mg/L of labeled α-ketobutyric acid (methyl-¹³C, 99%; 3,3-D₂, 98%; Cambridge Isotope Laboratories). For A_{2A}R cultures, add 4 mM dry theophylline (low-affinity antagonist) ten minutes prior to induction, and induce with 0.5% d₄-methanol. Reduce the temperature to 20 °C after induction. As with the small test cultures, add additional aliquots of 0.5% d₄-methanol roughly every 12 hours to maintain expression levels. Harvest cells after 36–48 hours and store at –80 °C. See Fig. 2 for a schematic of the deuterated media adaptation procedure.

- a. **Note 1:** Sterility should be maintained as much as possible during adaptation due to the lack of antibiotics in the media. Typically, this requires a dedicated shaker for *Pichia* cultures and a clean flame area for inoculations and media transfers.
- b. **Note 2:** Instead of d₈-glycerol, deuterated glucose can be used as an alternative (and somewhat less expensive) carbon source in *Pichia* cultures (Emami, Fan, Munro, Ladizhansky, & Brown, 2013; Fan et al., 2015; Fan, Shi, Ladizhansky, & Brown, 2011). However, glucose is a very strong repressor of the AOX promoter, thus any residual glucose in the cells or media can result in toxic conditions during induction (Wood & Komives, 1999).
- c. **Note 3:** Deuterated media can be saved and recycled to offset some of the costs of deuteration. Recycling can be done in-house by flash distillation (Moore, 1979) or shipped to Cambridge Isotope Laboratories for their D₂O recovery program (<https://www.isotope.com/sales-technical-support/D2O.cfm>).

Purification/NMR sample preparation

During optimization of culture and expression conditions (prior to purification), it is important to validate expression of A_{2A}R or other target proteins via western blots using specific antibodies to the protein or affinity tag(s). In our experience, a 10–20 µL volume of cells from the final harvested culture is typically sufficient to obtain a robustly-visible signal with an anti-His₆ antibody (Pierce) for A_{2A}R or other His-tagged membrane proteins. Once an optimized level of expression is demonstrated, the receptor can be purified from *Pichia* pellets following the procedure described below. Ideally, the purification should be “straight-through,” without any substantial delays from cell disruption to final sample; if required, the affinity-purified receptor can be flash-frozen in liquid N₂ prior to SEC with minimal (10–15%) losses of final yield.

Equipment

Stir plate

Glass Dounce homogenizer with loose and tight plungers

Refrigerated ultracentrifuge (100,000+ rcf)

Orbital shaker

FPLC with Superdex200 or comparable size exclusion column

Buffers

1. Lysis buffer: PBS containing 10% glycerol, 4 mM theophylline (low-affinity antagonist for A_{2A}R), 2 mM EDTA, and protease inhibitors (160 µg/mL benzamidine, 2.5 µg/mL leupeptin, 1 mM PMSF, 1 µM E-64)
2. Hypotonic buffer: 10 mM HEPES pH 7.5, 2 mM EDTA, 4 mM theophylline, protease inhibitors as above.
3. Solubilization buffer: 500 mM NaCl, 50 mM HEPES pH 7.5, 20% glycerol, 1% w/v DDM (Anatrace), 4 mM theophylline, and protease inhibitors as above.
4. TALON equilibration buffer: 250 mM NaCl, 50 mM HEPES pH 7.5, 0.05% w/v protonated DDM, 5% glycerol, 4 mM theophylline, and 30 mM imidazole.
5. Buffers A-E: 250 mM NaCl, 50 mM HEPES pH 7.5, 5% glycerol, 20 mM imidazole, ligand of interest (e.g., 10 µM of the A_{2A}R antagonist ZM241385 or 20 µM of the agonist NECA), made in D₂O, and containing 0.05% w/v DDM in the following protonated:deuterated ratios: (A) 4:0; (B) 3:1; (C) 2:2; (D) 1:3; (E) 0:4.
6. SEC buffer: 150 mM NaCl, 20 mM HEPES pH 7.5, 0.05% w/v deuterated DDM, ligand of interest (e.g., 10 µM ZM241385 or 20 µM NECA), 99% D₂O. Store at 4° C after 0.2 µm sterile filtering.

Procedure

Day 1

1. Thaw cell pellets and resuspend in 200–300 mL cold lysis buffer. All volumes in this protocol are given for a culture of 1 L, and should be scaled appropriately for different volumes.
2. Lyse cells with a high-pressure microfluidizer (Microfluidics M-110P or similar), using three passes at 24,000 psi, allowing the lysate to cool between passes. Pack ice or ice packs around microfluidizer coils to minimize sample heating.
3. To digest yeast cell wall glycans, add zymolyase (LongLife Zymolyase from G-Bio Sciences or similar) to 15 U/mL and incubate at 37 °C for 1 hour with stirring.
4. Separate out total membranes by centrifugation at 140,000 rcf for 30 minutes. Carefully decant the supernatant and keep membrane pellets on ice.
5. To wash membranes, resuspend the pellets in 200–300 mL of cold lysis buffer using a Dounce homogenizer (loose, 10 passes), then centrifuge at 140,000 rcf for 30 minutes again. Carefully decant the supernatant and keep membrane pellets on ice.
6. To lyse any intact spheroplasts and further wash the membranes, resuspend the pellets in 200–300 mL of cold hypotonic buffer using a Dounce homogenizer (loose, 10 passes). Incubate at 4 °C for 30 min with stirring, then centrifuge at 140,000 rcf for 30 minutes. Carefully decant the supernatant and keep membrane pellets on ice.
7. To solubilize membranes, resuspend the pellets in cold solubilization buffer using a Dounce homogenizer (loose, 10 passes; tight, 30 passes) and incubate at 4 °C for 2 hours with stirring. Centrifuge at 140,000 rcf for 30 minutes to remove insoluble material.
 - a. **Note:** The addition of cholesteryl hemisuccinate (CHS) to the micelles as a cholesterol analogue has been demonstrated to increase the stability and activity of A_{2A}R and other GPCRs (Liu, Chun, Thompson, Chubukov, Xu, Katritch et al., 2012; Weiss & Grishammer, 2002). We initially solubilized and purified A_{2A}R in DDM buffer with CHS included at a concentration of 0.2% and 0.01% w/v, respectively (Fig. 3A). While the inclusion of CHS improved NMR sample stability (Fig. 3B), it gave rise to an intense methyl peak at around 25 ppm ¹³C/0.85 ppm ¹H that interfered with analyses of NMR spectra (particularly relaxation measurements detailed below, which were extremely difficult to analyze until CHS was removed from the preparation). We note that a small peak at the same chemical shift remained in our CHS-free samples (Fig. 3A), suggesting that the receptor copurified with a cholesterol-like molecule.

8. During the final centrifugation step, pre-equilibrate 10–15 mL of fresh immobilized cobalt resin (such as TALON resin from Clontech) in TALON equilibration buffer. After centrifugation, decant supernatant into 250 mL tubes and add 30 mM imidazole to reduce background nonspecific binding. Incubate the supernatant with the equilibrated resin and bind overnight at 4 °C with gentle agitation.

Day 2

1. Spin down loose TALON resin at 2000 rcf for 10 minutes, remove the majority of the unbound material, and transfer to a gravity flow column. Through a series of wash steps, exchange the protonated DDM for deuterated DDM and simultaneously exchange theophylline (low-affinity antagonist) for a high-affinity ligand of interest (or no ligand). This is achieved by washing resin sequentially with 4 column volumes (CV) each of buffers A-E. Wash steps should be carried out at 4 °C and at a flow rate of 1–1.5 mL/min to ensure efficient exchange.
2. Elute tagged protein with Buffer E + 250 mM imidazole. A_{2A}R should elute within 2 CV. If necessary, purified A_{2A}R can be snap frozen in liquid N₂ in a buffer containing glycerol at a final concentration of 15% (e.g. addition of 10% glycerol to eluate).
 - a. **Note:** Another option for solution state NMR is reconstituting the proteins into nanodiscs, in which the protein is surrounded by lipids held together by an apolipoprotein band (Bayburt & Sligar, 2002, 2010; Denisov, Grinkova, Lazarides, & Sligar, 2004; Puthenveetil, Nguyen, & Vinogradova, 2017). We carried out experiments in which A_{2A}R was reconstituted into deuterated DMPC nanodiscs containing 10% cholesterol. While these samples exhibited a substantially-improved stability under NMR conditions, this advantage was negated by the presence of many intense lipid-derived peaks in the methyl region which significantly complicated spectral analysis. As a result, we decided to use deuterated DDM micelles as our membrane mimetic. For further discussion of nanodisc use in NMR, see also chapter “Synthetic biology-based solution NMR studies on membrane proteins in lipid environments” by Henrich et al.
3. Concentrate eluted protein using 100 kDa MWCO centrifugal concentrators to 1 mL for injection on a Superdex200 or comparable column (e.g., S200 GL Increase 10/300). As with many other membrane proteins, it is important to frequently resuspend the sample during concentration and spin at a slow speed (<3500 rcf) to reduce aggregation.
4. Pre-equilibrate Superdex200 column with SEC buffer, inject concentrated protein, and collect elution peak. The peak should elute at a volume commensurate with monomeric receptor with bound micelle.

- a. **Note:** Sodium has been shown to be an allosteric modulator of A_{2A}R and other Class A GPCRs (Gao & Ijzerman, 2000; Liu et al., 2012). We obtained high-quality spectra of A_{2A}R in the presence of 150 mM NaCl, although the minimal chemical shift differences observed between agonist- and antagonist-loaded samples suggest that cation choice has a substantial effect in this system. In contrast, purification in 150 mM KCl led to more heterogeneous spectra and less stable samples that were resistant to relaxation experiments and analysis, although there was a clear difference between agonist- and antagonist-bound spectra under these conditions (Clark et al., 2017).
5. Concentrate eluted protein using 100 kDa MWCO centrifugal concentrators to ~100 μ L for NMR samples. Frequently resuspend sample and spin at a slow speed (<3500 rcf) to reduce aggregation. Using 3 mm Shigemi tubes (100 μ L volume) allows us to concentrate samples to a concentration of ~100 μ M, leading to adequate signal-to-noise ratios for relaxation data.

Final Note: After establishing a purification protocol, it is important to verify that the protein sample is functional. In the case of GPCRs, the standard readout is coupling to G-protein. This is achieved by liposome reconstitution and G-protein activation assays (Cerione & Ross, 1991) and/or G-protein complex formation in detergent followed by subsequent gel filtration chromatography steps (Rasmussen, DeVree, Zou, Kruse, Chung, Kobilka et al., 2011). For A_{2A}R, we verified functionality with both methods (Clark et al., 2017) and found that the purification yielded a high level of functional receptor that can efficiently couple to the G_s heterotrimer.

Running ¹³C/¹H HMQC and methyl relaxation experiments

Once the protein sample is generated, the protein can be analyzed by NMR. A ¹³C/¹H HMQC experiment reports on overall folding and stability of the protein sample, as well as the effects of any perturbations, such as addition of binding partners. To be able to interpret the NMR spectra, some or all of the methyl peaks must be assigned to the residues that they arise from. Finally, residue-specific dynamics at various time scales can be measured by specialized NMR experiments. Here, we focus on our application of a methyl relaxation experiment measuring the fast (psec-nsec) sidechain motions (Sun et al., 2011; Tugarinov, Sprangers, & Kay, 2007) and adaption to short-lived membrane protein samples.

Equipment and Software

High field NMR spectrometer with cryoprobe (e.g. Bruker AVANCE III HD 800 MHz with TCI probe)

3 mm solvent-matched Shigemi NMR tube

NMRpipe software (Delaglio, Grzesiek, Vuister, Zhu, Pfeifer, & Bax, 1995)

Python installation with NumPy and SciPy modules

Procedure

1. Load the sample into an NMR tube (e.g. 3 mm solvent-matched Shigemi tube). Insert tube into spinner and spinner into high field NMR spectrometer with cryogenically-cooled probe.
2. Set temperature to 30 °C and allow to equilibrate for ~5 minutes.
3. Lock and shim the sample, tune the probe head for ^1H and ^{13}C , and determine the ^1H 90° pulse width as per usual NMR protocols.
4. Acquire a 1D ^1H spectrum to look for detectable isoleucine methyl signals (approx. -0.5 to 1.2 ppm) that are not swamped by other signals (buffer, detergent, lipids).
5. Acquire a $^{13}\text{C}/^1\text{H}$ HMQC (Ollerenshaw, Tugarinov, & Kay, 2003) spectrum centered on the isoleucine methyl region to assess sample quality, stability, and degree of secondary and tertiary structure.
 - a. **Note 1:** The optimal spectral parameters may vary a bit from sample to sample. The ^{13}C and ^1H carrier frequencies, spectral widths, and number of points collected for our A₂A_R samples are shown in Table 1. For a new sample, it is recommended to collect a spectrum with increased spectral width in the ^{13}C dimension (e.g., 40 ppm centered at 20 ppm) to determine where all isoleucine $\delta 1$ methyl peaks appear and where other signals arising from the sample (e.g., lipid, detergent, buffer, ligand) may fall. This initial spectrum can then be used to design a combination of spectral width and center frequency that avoids aliasing in contaminating peaks over the peaks of interest.
 - b. **Note 2:** Wild-type eukaryotic membrane proteins, and especially GPCRs, have limited stability in the micelle-solubilized form required for obtaining well-resolved spectra in solution state NMR studies. As shown in Fig. 3B, the intensities of several A₂A_R signals dropped significantly at ~12 hours. With this in mind, it is highly recommended to collect consecutive somewhat short (1–2 hr) HMQC experiments, process them independently, and look whether peaks are decreasing or changing radically with time. Those experiments that result in similar spectra can then be summed and processed together to increase the signal-to-noise ratio. NMRpipe (Delaglio et al., 1995) offers simple commands (“addNMR”) for summing NMR data.
6. To assign isoleucine $\delta 1$ methyl peaks, generate mutants in which a single isoleucine has been mutated to a different residue type, then express, purify, and collect a $^{13}\text{C}/^1\text{H}$ HMQC spectrum on each as described above. Ideally, one peak will disappear and that peak can be assigned to the mutated residue.
 - a. **Note:** This method relies on the mutation perturbing as little of the overall protein structure as possible. Thus, it is important to choose residues to mutate (and thus assign) that are biologically interesting but

not indispensable for the proper folding of the protein. The identity of the mutant also plays a large role; we have found that in general isoleucine to valine mutations work well; however, in certain cases it may be necessary to test multiple mutants, such as leucine or methionine (Fig. 4). This is often the most time- and cost-limiting step of a study. It is recommended to limit assignments to the most interesting or important residues.

7. On the same, or ideally on another freshly-prepared sample, acquire relaxation measurements. Here we focus on the relaxation violated coherence-transfer experiment and its measurements of methyl order parameters (S^2_{axis}) which report on fast (psec-nsec) motions of the side-chains (Sun et al., 2011; Tugarinov et al., 2007). The experiment and analysis are outlined in Fig. 5A. Additional discussion of the practical aspects of collection and analysis of methyl relaxation data is presented in Chapter “Characterization of internal protein dynamics and conformational entropy by NMR relaxation” by Stetz et al. Other experiments for measuring slower (μsec -msec) dynamics exist, but will not be detailed here (Korzhnev, Klobner, Kanelis, Tugarinov, & Kay, 2004).
 - a. **Note 1:** Relaxation experiments rely on collecting multiple spectra from experiments which differ solely by the duration of a relaxation delay and fitting spectral peak intensities as a function of this delay to the appropriate equation to extract relaxation parameters which in turn provide insights into dynamics. It is important to collect sufficient data points to generate a good fit (number of delays) and measure a long enough delay for the (usually exponential) function to decay (longest delay). These values vary based on the size of the particle and rotational tumbling time. For the class of experiments described here, a recommended set of delays for ~100 kDa particle is 0.8, 2, 3, 4, 6, 8, 10, 14 msec (Sun et al., 2011). However, since a pair of forbidden and allowed experiments needs to be collected for each delay in this class of relaxation experiment, this large number of time delays can lead to long experimental times, which may be problematic for samples with relatively short lifetimes (see below).
 - b. **Note 2:** In our experience, the combination of sample concentration and stability of the membrane protein sample significantly limited our ability to collect a full set of sixteen spectra as described in **Note 1**. As detailed above (**Note 2** on **Step 5** and Fig. 3B), our samples had a useful lifetime of 12–14 hours in the spectrometer at 30 °C, limiting the number of relaxation delays that could be tested. To overcome this, we modified the experimental setup to collect allowed experiments (the higher signal-to-noise experiment) at 5 relaxation delays and a forbidden experiment with 5 times the number of scans at 1 relaxation delay. The choice of forbidden relaxation delay is important; an intermediate value (for us, 8 msec) that has good peak intensity for both forbidden and allowed spectra is highly preferable. The pulse sequence

was modified accordingly and the experiment was processed using a custom-written NMRpipe (Delaglio et al., 1995) macro to appropriately split the experiments. Both pulse sequence and macro are available upon request. This change requires modifying the analysis and fitting protocol (see **Note 2** on **Step 10** below).

8. This relaxation experiment is implemented as a pseudo-4D experiment with alternating forbidden/allowed experiments and relaxation delays as the third and fourth dimensions, respectively. To process relaxation experiments, either extract the 2D spectrum at each relaxation delay (allowed and forbidden) using the Bruker TopSpin **rser2d** command and process each dataset as an individual 2D spectrum, or use a NMRpipe script that iterates over the relaxation delays to simultaneously split and process (a sample can be provided on request). NMRpipe (Delaglio et al., 1995) and NMRFx Processor (Norris, Fetler, Marchant, & Johnson, 2016) are both good options to process extracted 2D datasets.
9. To analyze the relaxation data, first extract peak intensities for each peak to be analyzed for each relaxation delay (allowed and forbidden) using an analysis program such as NMRViewJ (Johnson, 2004). To estimate peak intensity errors in NMRViewJ, either select an empty region of the spectrum and measure the standard deviation of signal across it (in the **Analysis** tab in NMRView J), or use the command **nv_dataset noise data_name.nv** in the NMRViewJ console. This noise estimate may be different for each plane or spectrum and will be used for error analysis of the fit.
10. To extract relaxation parameters, fit the ratio of forbidden to allowed peak intensities to the following Equation 1 (Sun et al., 2011), where N is the number of scans for each experiment, T is the relaxation delay, δ (< 0) is a term for the coupling between rapidly and slowly decaying single-quantum coherences, and η is a relaxation rate:

$$\left| \frac{I_{forb}}{I_{all}} \right| = \frac{3N_{all}}{4N_{forb}} \frac{\eta \tanh(\sqrt{\eta^2 + \delta^2} T)}{\sqrt{\eta^2 + \delta^2} - \delta \tanh(\sqrt{\eta^2 + \delta^2} T)} \quad (1)$$

The two fit parameters are η and δ , which are constrained to be > 0 and < 0 , respectively, within the fit.

- a. **Note 1:** There are multiple approaches to estimate errors in the fit parameters. One is to propagate noise estimates into the intensity ratios, then use those errors to weight the data points during the least-squares minimization and determine the errors of the fit parameters from the covariance matrix. The **optimize.curve_fit** function in the SciPy Python module provides reasonably simple functionality for this process (Oliphant, 2007). Another approach is a **nonparametric Monte Carlo bootstrap** (Efron, 1981), in which for each set of datapoints to fit, many (100–1000) artificial datasets are simulated centered on the

measured peak intensities (and within error estimates from the noise) and then fit individually. The mean and standard deviation of the fit parameters provide the η and δ values and errors. A comparison of these two methods applied to data from A₂A_R Is shown in Fig. 5B; the error estimates in the bootstrap approach are sometimes larger and sometimes smaller than those from the covariance matrix.

- b. Note 2:** For the modified relaxation experiment with only one forbidden relaxation delay (together with a complement of several allowed relaxation spectra) described above in **Note 2** on **Step 7**, the processing step (**Step 8**) should be modified to correctly split allowed experiments from the one forbidden experiment. A sample NMRpipe script can be provided upon request. Peak intensity quantification and error estimates (**Step 9**) are unaffected. The analysis (**Step 10**) involves simultaneous fitting of the single forbidden to allowed intensity ratio to Equation 1 and all allowed peak intensities to the following Equation 2 (Sun et al., 2011), where A is a constant, T is the relaxation delay, and $R_{2,H}^S$ and $R_{2,H}^F$ are the slow and fast relaxation rates of methyl proton single-quantum transitions:

$$I_{all} = A \frac{3}{2} [\exp(-R_{2,H}^S T) + \exp(-R_{2,H}^F T)] \quad (2)$$

Unfortunately, this approach introduces more fit parameters: A, $R_{2,H}^S$ and $R_{2,H}^F$ (all of which were constrained to be > 0). Using data collected on *E. coli* maltose binding protein as a test case, we achieved good correspondence between η values calculated from 5 pairs of allowed and forbidden experiments and from 5 allowed and 1 forbidden experiment (Clark et al., 2017). In the case of A₂A_R, the isoleucine residue in the flexible C-terminal Protein C tag served as a serendipitous control with markedly reduced relaxation rate than the other residues.

- 11.** The value of η can be used to extract the S_{axis}^2 order parameter of the methyl group using the following Equation 3 (Sun et al., 2011), where τ_c is the molecular rotational correlation time, μ_0 is the vacuum permittivity constant, γ_H is the proton gyromagnetic ratio, r_{HH} is the distance between pairs of methyl protons (estimated as 1.813 Å), the function $P_2(x) = 1/2(3x^2 - 1)$, and $\theta_{axis,HH}$ is the angle between the methyl axis and a line between two methyl ¹H nuclei (90°) (Tugarinov & Kay, 2006):

$$\eta = \frac{9}{10} \left(\frac{\mu_0}{4\pi} \right)^2 [P_2(\cos\theta_{axis,HH})]^2 \frac{S_{axis}^2 \gamma_H^4 \hbar^2 \tau_c}{r_{HH}^6} \quad (3)$$

The rotational correlation time τ_c can be measured using a ¹⁵N/¹H-TRACT experiment (D. Lee, Hilty, Wider, & Wuthrich, 2006) or ¹⁵N T₁/T₂ relaxation analysis (Farrow, Muhandiram, Singer, Pascal, Kay, Gish et al., 1994). However,

like all global estimates of tumbling time, these methods may underestimate the rotational correlation time due to contributions from fast internal dynamics.

Final Note: Pulse sequence, macros, and scripts all available on request.

Concluding remarks

As detailed here, we have developed a protocol using α -ketobutyrate (the keto acid precursor for isoleucine) supplementation of the growth media for *Pichia pastoris*, to prepare Ile $\delta 1$ methyl-labeled GPCRs (Clark et al., 2017; Clark et al., 2018; Clark et al., 2015). This protocol is analogous to methods previously developed for expression and labeling in *E. coli* (Gardner & Kay, 1997); however, it allows access to integral membrane proteins such as GPCRs that cannot be efficiently expressed in a prokaryotic host. The samples we have generated are suitable for a range of solution NMR measurements, including relaxation-based determination of ligand-dependent changes in receptor dynamics. These data complement other NMR-based measurements of receptor structure, dynamics, and interactions using probes at other ^{13}C methyl groups (Kofuku et al., 2014; Kofuku et al., 2018; Solt, Bostock, Shrestha, Kumar, Warne, Tate et al., 2017), exogenously added ^{19}F probes (Ye, Neale, Sljoka, Lyda, Pichugin, Tsuchimura et al., 2018; Ye, Van Eps, Zimmer, Ernst, & Prosser, 2016), or $^{15}\text{N}/^1\text{H}$ pairs in receptors stabilized by point mutations or insertion into covalently-circularized nanodiscs (Isogai et al., 2016; Nasr, Baptista, Strauss, Sun, Grigoriu, Huser et al., 2017).

Taken together, these studies show the rich diversity of GPCR conformations and dynamics, taking advantage of the power of solution NMR to get site-specific information from receptors in equilibrium and complementing structural studies from X-ray diffraction and cryo-EM. In addition, we believe that, properly modified, these approaches can be applied to study the dynamics and interactions of other eukaryotic membrane proteins as well. We anticipate that further technological developments, including those in sample preparation [e.g., additional labeling strategies (Robson, Takeuchi, Boeszoermyenyi, Coote, Dubey, Hyberts et al., 2018), ways to improve sample stability or concentration via improved membrane-mimetics such as smaller, more “NMR-friendly” nanodiscs (Chien, Helfinger, Bostock, Solt, Tan, & Nietlispach, 2017; Hagn, Etzkorn, Raschle, & Wagner, 2013)], NMR data acquisition [e.g., ultrahigh field NMR systems (Quinn, Wang, & Polenova, 2018), improved $^{13}\text{C}/^{15}\text{N}$ direct detection methods (Takeuchi, Heffron, Sun, Frueh, & Wagner, 2010)], and analysis [e.g., improved methods for chemical shift assignment through PRE or NOESY experiments (Lescanne, Skinner, Blok, Timmer, Cerofolini, Fragai et al., 2017)], will only further improve such prospects in the future.

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Figure 1: Optimization of MF α signal sequence.

A) and B) General schematics of WT and optimized MF α signal sequences prior to start of WT GPCR gene of interest. As shown in alignments in C), six mutations (V22A, G40D, L42S, V50A, V52A, and F55L) plus a deletion of residues 57–70 generate an optimized version of the MF α signal sequence (“MF α Opt”) that was used for efficient A_{2A}R expression at the plasma membrane.

Figure 2: Adaptation of *Pichia pastoris* to deuterated media.

Summary of schemes used to adapt *Pichia* cultures to increasingly deuterated media to ensure efficient deuteration levels and protein expression. See section on Expression and isotopic labeling in *Pichia* for more details.

Figure 3: Receptors reconstituted with CHS are more stable but have crowded spectra.

A) $^{13}C/^1H$ HMQC spectra of $A_{2A}R$ in DDM micelles with CHS (left) and without added CHS (right). Reconstitution with CHS resulted in spectra with intense extraneous signals, likely arising from lipids, as well as a very intense cholesteryl methyl peak (dark gray). In DDM micelles without added CHS, the dispersion of protein-derived peaks is comparable, but there are fewer extraneous peaks in the region. A weak signal at the CHS/cholesterol methyl chemical shift suggests that some sterol was copurified with the protein. B) $^{13}C/^1H$ HMQC spectra of $A_{2A}R$ in DDM micelles with CHS (left) and without added CHS (right) collected after different lengths of time at 30 °C. Several protein signals in the DDM micelles without CHS drop significantly after 14 hours (dark orange).

Figure 4: Assignment of selected A_{2A}R Ile-81 methyl peaks by site-directed mutagenesis. ¹³C/¹H HMQC spectra of wild-type A_{2A}R (black) are shown overlaid with four mutants (purple). In some cases, such as I238, only one peak disappeared and was assigned (blue circle). In others, such as I274, manifold spectral changes were present, and multiple mutants were needed, along with solvent PRE data (Clark et al., 2017) to make the assignment (blue circle). In other cases, such as I106, the mutation completely abrogated protein folding or stability.

Figure 5: Schematic of fast motions relaxation experiment.

A) Sample $^{13}\text{C}/^1\text{H}$ planes at the indicated relaxation delays for *E. coli* MBP are shown to illustrate the decay of allowed signal intensity and buildup of forbidden signal intensity. To derive relaxation rate η , peak intensities are extracted and the forbidden:allowed ratio is fit to Equation 1 as a function of relaxation delay. B) Plot of extracted η relaxation rates and their errors using either a Monte Carlo bootstrapping approach with 1000 selected datasets (red/yellow) or covariance matrix of the fit (blue/cyan) for A₂A. Ile-51 methyl peaks are listed by an arbitrary peak ID number.

Table 1:

Typical spectral parameters for $^{13}\text{C}/^1\text{H}$ HMQC spectra of $\text{U-}^2\text{H}$ (^{13}C , ^1H Ile $\delta 1$ CH_3) labeled GPCR samples.

Nucleus	Center Frequency (ppm)	Spectral Width (ppm)	Number of Points	Acquisition Time (msec)
^1H	4.7	13.35	1024	7
^{13}C	14	22	64	48