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Expression and NMR Structural Studies of Isotopically Labeled Cannabinoid Receptor Type II

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Abstract

Cannabinoid receptor type II (CB₂) is an integral membrane protein with seven transmembrane helices that belongs to the large superfamily of rhodopsin-like G protein-coupled receptors (GPCR). The CB₂ is part of the endocannabinoid system that plays a vital role in regulation of immune response, inflammation, pain, and metabolic processes. Information about the structure and function of CB₂ in cell membranes is essential for development of specific pharmaceuticals that target CB₂ signaling. Methodology for recombinant expression, stable isotope labeling, purification, reconstitution into liposomes, and NMR characterization of functionally active CB₂ is presented. Uniformly ¹³C, ¹⁵N-labeled CB₂ protein is expressed by fermentation of *E. coli* in a medium of defined composition under controlled aeration, pH and temperature and purified by tandem affinity chromatography. The receptor reconstituted into lipid bilayers is suitable for structural studies by solid-state NMR spectroscopy at low temperature.

Keywords

cannabinoid receptor CB₂; bacterial fermentation; G protein-coupled receptor; nuclear magnetic resonance; stable isotope labeling; affinity purification; recombinant protein

1. INTRODUCTION

Recent success with studying the structure of GPCR by crystallography has stimulated interest in investigating structure and function of GPCR in their natural environment, the lipid bilayer, which is achieved by NMR spectroscopy. Here we describe methods of expression, purification, reconstitution and solid-state NMR investigation of cannabinoid receptor type II, CB₂. The CB₂ receptor is primarily located in cells of immune and hematopoietic systems such as thymus, spleen, and tonsil (Cabral & Griffin-Thomas, 2009). Expression of CB₂ in neuronal microglia was reported (Fernandez-Ruiz et al., 2007; Nunez et al., 2004) but the topic continues to be discussed controversially. The discovery of the endogenous cannabinoid ligands anandamide and 2-AG stimulated studies of the intrinsic

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³This is manifested by the rapid increase in the concentration of the dissolved oxygen. The absence of glucose in the medium is confirmed also by direct measurements using a glucose meter.

¹¹**Safety note:** Route the cold air emitted from the MAS rotor out of the magnet through a plastic pipe to avoid cooling the room temperature shims and bore tube of the magnet below specifications provided by the manufacturer. Flush the probe and the shim set with a high stream of dry air and monitor the temperature of the shim set to avoid damaging the magnet.

function of cannabinoid receptors (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). The CB₂ receptor plays a major role in inflammatory processes in a variety of organs including liver, kidneys and the gastrointestinal system (Pacher & Mechoulam, 2011). Migration behavior of immune cells upon activation of the receptor has also become the subject of deep interest (Miller & Stella, 2008). The need for novel treatments of inflammation and pain has encouraged development of selective ligands for CB₂ receptor that are free from the psychoactive side effects of conventional phytocannabinoids (Ibrahim et al., 2003; Nettekoven et al., 2016).

The receptor inhibits adenylyl cyclase via G_{iα}/G_{oα} protein subunits, while G_{βγ} subunits signal via mitogen-activated protein kinases (MAPK) and extracellular signal-regulated kinases (ERK) pathways. Deactivation and internalization of CB₂ occurs via G protein-coupled receptor kinases that facilitate arrestin binding and receptor internalization.

To study structure and function of CB₂ in membranes by NMR, expression and functional reconstitution of highly purified and homogeneous receptor into membranes or membrane-mimetic environments of controlled composition in the range of milligrams is required (A. Yeliseev, Zoubak, & Gawrisch, 2007; A. A. Yeliseev, Wong, Soubias, & Gawrisch, 2005). Structural instability of GPCR in detergent micelles, and to a lesser degree in membranes, is a well-known phenomenon that requires special attention during expression, purification and reconstitution (Vukoti, Kimura, Macke, Gawrisch, & Yeliseev, 2012). Therefore, reconstitution of GPCR must be followed by functional studies to assure preservation of structural integrity.

Furthermore, for NMR experiments, the receptor must be efficiently labeled with NMR active, stable isotopes like ¹³C and ¹⁵N. NMR experiments may take from hours to weeks per sample for completion. Experimental conditions must be found that prevent deterioration of the protein during the time-course of experiments (Berger et al., 2010; Kimura et al., 2014; Kimura et al., 2012).

2. PREPARATION OF STABLE ISOTOPE-LABELED CANNABINOID RECEPTOR – AN OVERVIEW OF THE METHOD

The isotopically labeled protein is produced by fermentation of the *E. coli* strain expressing CB₂ in a minimal salt medium (MSM). The bacterial culture is grown in a fermenter under controlled pH, aeration conditions and temperature, with supplementation of the medium with stable isotope-labeled sources of carbon (¹³C-glucose) and nitrogen (¹⁵NH₄Cl). The plasmid-based expression construct for CB₂ consists of the N-terminal maltose binding protein (MBP) expression partner, a recognition site for specific tobacco etch virus (TEV) protease, as well as two affinity tags, twin-Strep-tag and His₁₀ tag, for chromatographic purification (see Fig. 1A). The CB₂ receptor accumulated in cell membranes is solubilized in detergents and purified by two steps of affinity chromatography on Ni-NTA and Strep-Tactin resins (A. Yeliseev et al., 2007). The N-terminal expression partner MBP is removed by cleaving the fusion with TEV protease. The purity of the protein is assessed by SDS-PAGE and by the Western blot, and yield by the modified Lowry protein assay (Thermo Fisher Scientific). The functional activity of the protein is established by a G protein-activation

assay. The affinity purification of CB₂ protein is summarized in Fig. 1. The purified protein is then reconstituted into proteoliposomes that are transferred to a rotor for solid-state magic-angle spinning ¹³C-, ¹⁵N NMR experiments.

3. Materials

The synthetic cannabinoid agonist, CP-55,940 ((-)-*cis*-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol) is from Tocris Bioscience (Ellisville, MO). ³H-labeled CP-55,940 and [³⁵S]-GTPγS are from Perkin Elmer (Waltham, MA). The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine sodium salt (POPS) used for CB₂ receptor reconstitution are purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate-Tris salt (CHS) is from Anatrace (Maumee, OH). The fluorophore-labeled lipid 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate salt (DiI_{C18}(5) solid) is from Thermo Fisher Scientific. The detergents 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and *n*-dodecyl-β-D-maltoside (DDM) are from Anatrace. ¹⁵NH₄Cl (99% enrichment) and D-glucose (U-¹³C₆, 99% enrichment) are from Cambridge Isotope Laboratories (Andover, MA).

4 Expression of CB₂ in a Fermenter

4.1 Composition of the Mineral Salt Medium

The MSM, slightly modified from conditions used earlier (Berger et al., 2010), contains: 4.65 g/L Na₂SO₄, 14.6 g/L K₂HPO₄, 4.07 g/L NaH₂PO₄ x 2H₂O, 1.2 g/L MgSO₄ x 7H₂O, 3.32 mg/L CaCl₂ x 2H₂O, 0.72 mg/L ZnSO₄ x 7H₂O, 0.4 mg/L MnSO₄ x H₂O, 69.48 mg/L EDTA, 40.1 mg/L FeCl₃, 0.236 mg/L CuSO₄ x 5H₂O, 0.84 mg/L CoCl₂ x 6H₂O. Supplement with 10 g/L glucose, 2.74 g/L NH₄Cl, 100 mg/L thiamine hydrochloride and 100 mg/L ampicillin prior to use.¹

4.2 Adaptation of Cells to MSM

- Inoculate 5 mL of MSM with 50 μL of glycerol stock of *E. coli* cells BL-21(DE3) grown in LB medium, harboring plasmid pAY130 (A. Yeliseev et al., 2007). Grow cells overnight.
- Prepare a new glycerol stock by mixing 1 mL of cell culture with 1 mL of sterile 50% glycerol.
- Use 50 μL of this newly prepared glycerol stock to start fresh 5 mL MSM culture. The adaptation of cells to MSM is repeated at least two more times so that the cells in the final stock undergo a minimum of three adaptation cycles. The glycerol stocks should be stored at -80 °C until use.

¹Sterilize separately by autoclaving: phosphate salts, glucose, magnesium salts, ammonium chloride. Sterilize separately by filtration: calcium salts, microelements, thiamine and ampicillin.

4.3 Preparation of the Fermenter

Fermentation is carried out in a 3.0 L BioFlo[®] 110 Bench-Top Fermenter (New Brunswick Sci. Co., Edison, NJ) in a volume of 1 L.² Schematic representation of the fermentation process is presented in Fig. 2.

- Sterilize phosphate salts, magnesium salt, calcium salt, microelements, vitamins, glucose and ammonium separately. ¹⁵NH₄Cl is used throughout the fermentation.
- Sterilize 3L glass vessel filled with 500 mL of water.
- Add salts, microelements, vitamins, glucose and ammonium on the day of the fermentation. The concentration of NH₄Cl is 2.73 g/L and glucose – 10 g/L, respectively, and is adjusted throughout the fermentation as needed. Adjust the volume to ≈800 mL, to allow for additional 200 mL of seed culture for a total volume of 1 L.
- Adjust pH to 7.0.
- Set the temperature to 37 °C.
- Calibrate the oxygen electrode. Set the concentration of dissolved oxygen at 40% of saturating concentration. Set the control of dissolved oxygen by a cascade of stirring speed and flow of air.
- Calibrate the Assure[®] 3 Blood Glucose Monitoring System (Arkray, USA) according to the manufacturer's instructions using standard glucose solutions from 0.5 to 2.5 g/L.
- Calibrate the ammonia electrode with BNC connector (Thermo Scientific) according to the manufacturer's instructions using standard solutions of NH₄Cl from 1 to 5 g/L.

4.4. Fermentation in MSM

CB₂ fusion protein is expressed by fermentation of *E. coli* BL-21(DE3) cells harboring plasmid pAY130 (see Fig. 1A).

- Inoculate 25 mL of MSM supplemented with 100 µg/mL ampicillin in 125 mL shake flasks with 50 µL aliquot of MSM-adapted cells stored frozen in 25% (v/v) glycerol. Grow cells on a shaker at 230 rpm, at 37°C to OD₆₀₀ = 1–1.5.
- Use 4–5 mL of cell culture to inoculate 500 mL of MSM in a 2 L shake flask. Grow cells overnight to the OD₆₀₀ = 3.0–3.5. Collect cells by centrifugation at 3,000xg for 20 min in sterile 250 mL centrifuge bottles, and re-suspended in 200 mL of sterilized tap water.
- Inject cells into the fermenter so that the OD₆₀₀ of the culture at the start of fermentation is about 1.0.

²Fermentation can be performed in any fermenter with similar characteristics.

- Perform the cell cultivation as batch process. Adjust the concentration of glucose and ammonium chloride during the fermentation as needed. Take 1–2 mL samples from the culture every 30 minutes during fermentation to monitor cell growth.
- Monitor the concentration of glucose with the Assure® 3 Blood Glucose Monitoring System (Arkray, USA).
- Monitor the concentration of ammonium with Ammonia electrode with BNC connector (Thermo Scientific).
- Adjust the pH of the medium to 7.0 by the controlled addition of 10% NaOH and 10% H₃PO₄.
- When the OD₆₀₀ of the culture approaches 20, add the stock solution of CP-55,940 to the fermenter to the final concentration of 5 μ M.
- Reduce the temperature of the fermenter to 20°C.
- Induce production of the recombinant receptor by addition of filter-sterilized IPTG to the final concentration of 0.5 mM.
- After 2 hours of incubation, add another portion of IPTG to the final concentration of 1 mM.
- Continue fermentation for another 8 hours or until the OD₆₀₀ of the cell culture reaches $\approx$ 20.
- Collect cells by centrifugation at 5,000xg for 30 min at 4°C. Wash the cell pellet once with ice-cold PBS buffer. Collect the pellet by centrifugation at 5,000xg for 30 min and store at –80°C until use.

A schematic representation of the fermentation of *E. coli* expressing CB₂ is shown in Fig. 2.

5. Purification of Recombinant CB₂ Receptor

The CB₂-130 fusion protein, uniformly labeled with ¹³C and ¹⁵N, is extracted from the biomass with a mixture of detergents: DDM (1%, w/v), CHAPS (0.5% w/v) supplemented with CHS (0.1%, w/v) and ligand CP-55,940 (10 μ M). The addition of the ligand is necessary to ensure the stability of functional receptor through the purification procedure (Vukoti et al., 2012). All procedures should be performed on ice or at 4°C.

The N-terminal expression fusion partner MBP is removed by addition of the specific TEV protease taking advantage of the TEV recognition sequence downstream of MBP (Fig. 1, A). TEV protease can be obtained from commercial sources or is expressed in house. In this laboratory, it is prepared by expression of the *E. coli* BL21-RIL harboring plasmid pRK793 as described previously (Kapust et al., 2001; A. A. Yeliseev et al., 2005). Since the activity of protease is inhibited by high salt and glycerol concentrations, the fractions containing CB₂ protein are dialyzed against buffer containing reduced concentrations of glycerol and NaCl (A. Yeliseev et al., 2007).

The final step of protein sample preparation includes concentrating the protein in a spin-concentrator, which results in a co-concentration of some of the components of the elution buffer. Typically, the 40 μM CB₂ preparation contains DDM at a concentration of 0.4–0.6% (w/v), CHAPS 1.5–2% (w/v), CHS 0.4–0.6% (w/v), and 40–80 μM CP-55,940.

The purity of the isolated protein is assessed by SDS-PAGE (Fig. 1, B) and by Western blot probed with specific antibodies against CB₂ and affinity tags (A. Yeliseev et al., 2007).

- Prepare 400 mL of 12x concentrated stock solution of 6% (w/v) CHAPS - 1.2% (w/v) CHS.⁴
- Prepare 200 mL of 100 x concentrated stock solution of 10% (w/v) of DDM.
- Prepare 500 mL of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% DDM. Supplement the buffer with 10 μM CP-55,940. Filter through 0.45 μm sterile filter and store at 4°C.
- Prepare 200 mL of buffer B (buffer A supplemented with 250 mM imidazole). Adjust pH to 7.5.
- Prepare 200 mL of buffer C (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5% CHAPS, 0.1% CHS, 0.1% DDM). Filter through 0.45 μm filter and store at 4°C.
- Mix the biomass of the cells with 300 mL of ice-cold 50 mM Tris-HCl buffer, pH 7.5 supplemented with Complete, EDTA-free (Roche), 5 mM MgCl₂ and DNase I (Sigma) in a 1L blender.
- Disrupt the cells by passing the cell paste twice through the cell homogenizer (Avestin).
- Under continuous stirring add 500 mL of double-concentrated solubilization buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 60% (v/v) glycerol). Add 12x-concentrated stock solution of CHAPS/CHS and 10% DDM so that the final concentration of the detergents in 1L solution will be: 0.5% CHAPS, 0.1% CHS, 1% DDM.
- Add 20 mM stock solution of CP-55,940 in EtOH to the final concentration of the ligand 10 μM . Continue stirring on ice for 1h.
- Centrifuge solution in Ti-45 rotor at 43,000 rpm for 1 h. Collect the supernatant and filter through the 0.45 μm sterile filter.
- Apply the filtered solution at a flow rate 0.5–0.7 mL/min onto 5 mL Ni-NTA column pre-equilibrated with buffer A, on an AKTA-Purifier system or similar chromatography system that allows controlled application of the solution. Monitor the absorbance of the eluate at 280 nm.

⁴Prepare separately a solution of 24 g of CHAPS in 180–190 ml of water and a suspension of 4.8 g of CHS in 200 mL of water. Under continuous mixing, add dropwise the suspension of CHS to CHAPS solution. Continue mixing until solution becomes clear. Store at 4°C.

- Wash the resin with 20 column volumes of a mixture of 87% buffer A and 13% of buffer B.
- Elute protein by applying 100% buffer B over 7 column volumes. Collect 4 mL fractions.
- Combine fractions containing protein and dialyze for 1–2 h at 4°C in Dialyzer Mega tube with 30 kDa molecular weight cut-off (EMD Novagen) against buffer C.
- Transfer the content of the dialysis cassette to a 50 mL Falcon tube. Add 2 mg of purified TEV protease.⁵ Incubate at 4°C for 4 hours.
- Wash 4 mL of StrepTactin resin with 4x5 mL of buffer A. Add resin to the tube containing CB₂ and TEV protease and incubate overnight on a shaker.
- Transfer resin to a disposable plastic column and wash with 80 mL of buffer A.
- Elute CB₂ protein with 5x4 mL of 5 mM desthiobiotin in buffer A.⁶ Close the column after addition of every portion of elution buffer and incubate for 10 min.
- Combine elution fractions and concentrate on the Amicon Ultracel-30K spin concentrator to the final volume of 4–5 mL.
- Measure the protein concentration in the sample by a Bio-Rad DC protein assay kit.⁷ Aliquot the protein into Eppendorf tubes and snap-freeze in liquid nitrogen. Store the concentrated protein at –80°C until use.
- Analyze the purity of the protein sample by SDS PAGE and by Western blot with antibody against His-tag and CB₂ as described earlier (Vukoti et al., 2012).

6. Reconstitution of CB₂ into Lipid Bilayers

Reconstitution of the purified, uniformly ¹³C-, ¹⁵N-labeled CB₂ receptor into liposomes is performed by the rapid dilution method (Kimura et al., 2012). To accurately quantify the recovery of protein and lipid in the course of proteoliposome preparation, the protein sample can be supplemented with CB₂ protein labeled with Alexa Fluor 488, and a lipid mixture with fluorescently labeled lipid analog DiIC18(5).⁸ The protocol described here consistently allows reconstitution of CB₂ at approximately 1:600 protein-to-lipid ratio (mol/mol) with a yield of 80% or higher for protein, and 90% or higher for the lipid.

- Prepare 28 mg of a mixture of lipids POPC/POPS (4:1 mol/mol) and 5.6 µg of DiIC18(5) by mixing appropriate quantities of stock solutions of these lipids in organic solvents (typically methanol or chloroform) and remove the solvent under a stream of nitrogen or argon. Dissolve lipids in 0.5% CHAPS at a concentration of 3.3 mg/mL.

⁵TEV protease may be obtained from commercial sources or can be expressed and purified in house. The variant of protease used in this work contains a S219V mutation that makes it impervious to auto-inactivation, and a N-terminal His6 tag to facilitate purification.

⁶Weigh the necessary amount of desthiobiotin in an Eppendorf tube. Dissolve in a small volume of 1 N NaOH. Add the solution to buffer A, and adjust pH to 7.5.

⁷Add 5 µL of the elution buffer to standards in a microplate-based assay to account for differences in buffer composition.

⁸The quantification of protein and lipid by measuring fluorescence of the label is described in (Kimura et al., 2012).

- Supplement 2.8 mg of purified CB₂ protein with 60 µg of AlexaFluor 488-labeled CB₂.
- Mix protein with lipid on ice, in a total volume of 8.5 mL
- Dilute the mix dropwise into 650 mL of PBS buffer at 4°C under continuous stirring. Proteoliposomes are formed upon rapid dilution to below the critical micelle concentration (CMC) of the detergents.
- Remove detergent monomers formed upon dilution on a concentrating device (Amicon 8400: Millipore, Billerica, MA) using a polyethersulfone filter with a 30 kDa molecular mass cut off operated at 1 bar of Ar-gas pressure and at 4°C.
- Determine material recovery by measuring fluorescence of the labeled protein and lipids. Perform measurements on a Synergy HT Microplate reader (BioTek, Winooski, Vermont) or instrument with similar capabilities. Excite AlexaFluor at 488 nm and detect emission at 528 nm to measure protein content. To measure lipid content, excite the DiI C18(5) at 590 nm and detect emission at 645 nm.⁹
- Measure the functional activity of the recovered samples by determining the rates of G protein activation, as described earlier, using recombinant, purified G_{ai1} and G_{β1γ2} as described previously (Kimura et al., 2012; A. Yeliseev et al., 2007; A. Yeliseev et al., 2005).

7. ACQUISITION OF NMR SPECTRA

Solid-state magic-angle spinning (MAS) experiments have the advantage that experiments can be conducted directly on proteoliposomes with sufficiently high protein content (Kimura et al., 2014). The protein molecules must be in a highly homogeneous state. Samples can be studied at any temperature. However, sensitivity increases when proteins are immobilized which is conveniently achieved by lowering temperature. Lower temperatures are also beneficial for long-term survival of the protein.

The required protein content of samples depends on intrinsic resolution of resonances as well as the type of equipment that is used to record the spectra. A typical linewidth of ¹³C-, ¹⁵N NMR resonances of membrane incorporated peptides and proteins is 1 ppm (¹³C) and 2–3 ppm (¹⁵N), as observed for reconstituted CB₂ (Kimura et al., 2014) which translates into a minimal amount of 2–3 mg of CB₂ per sample for sufficient signal-to-noise on standard high-field NMR spectrometers operating at a protein resonance frequency in the range of 600 – 850 MHz. Final sample volume depends on the protein/lipid molar ratio of proteoliposomes and the residual water content of samples. Typical volumes of MAS samples are 30–50 µL, half of it buffer. Higher protein content greatly reduces acquisition times of NMR spectra and enables a broader range of experiments. On occasion, spectral resolution as low as 0.25 ppm for ¹³C resonances was reported, in particular for microcrystalline protein samples (Zech, Wand, & McDermott, 2005). Requirements for minimal sample size decrease proportionally with improved resolution.

⁹Cross-talk between the fluorophores was negligible at these settings.

7.1 NMR Sample Preparation

High-field NMR spectrometers have somewhat better performance if experiments are conducted on samples with low ionic content, e.g. 50 mM NaCl in buffer or lower. Proteoliposome may contain traces of paramagnetic ions, e.g. manganese ions from the mineral salt medium and nickel ions that leach from affinity columns, which shorten relaxation time of NMR resonances and broaden them at higher concentrations. Those ions are efficiently removed by immersing proteoliposomes in buffer containing 1–5 mM ethylenediaminetetraacetic acid (EDTA). Depending on the experiment, a low, controlled content of paramagnetic ions may reduce acquisition times of NMR spectra by allowing a shorter delay time between acquisitions of NMR signals.

- Pellet the proteoliposomes containing uniformly ^{13}C -, ^{15}N -labeled CB_2 receptor by ultracentrifugation at $400,000\times g$ or higher for 12 h at 4°C . Remove the supernatant and carefully blot away any remnants of buffer.
- A lossless transfer of the pellet to the rotor for MAS NMR is conveniently achieved by using plastic pipette tips cut to suitable dimensions to guide the pellet from the centrifuge tube to a suitable MAS rotor. Place the rotor/pipette tip/centrifuge tube setup into a swinging bucket centrifuge and transfer the pellet to the rotor by spinning at low speed.
- Hold the pellet in place with a Kel-F insert for liquid samples inside the rotor, e.g. 4 mm outer diameter zirconia rotor with Kel-F insert, effective sample volume $50\text{ }\mu\text{L}$.¹⁰

7.2 MAS NMR Experiments

Record the ^{13}C -, ^{15}N MAS NMR spectra of proteoliposomes on a spectrometer equipped with a 4-mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ MAS variable-temperature probehead, e.g. Bruker BioSpin, Inc.. Carry out the measurements at MAS frequencies in the range of 8 to 15 kHz, such that spinning sidebands do not superimpose on spinning center bands of interest. Choose a temperature of 4°C or lower for long-term survival of CB_2 (Vukoti et al., 2012).

- Spin sample (typical MAS spinning frequency 5–10 kHz) at a temperature slightly above the main phase transition temperature of lipids and shim magnetic field of the spectrometer on the well-resolved ^1H MAS NMR lipid resonances. Please note that spinning at frequencies higher than 5 kHz heats the sample above the temperature indicated on the spectrometer controller due to frictional heating in the MAS bearings. Thermally sensitive samples like CB_2 can be damaged if frictional heating is not taken into account. Lower the set temperature of the instrument accordingly to compensate for frictional heating. Relative changes of the chemical shift of the water resonance compared to lipid resonances are a good measure of changes in sample temperature.
- Lower the sample temperature to the desired value while keeping the sample spinning at the desired frequency. For CB_2 with excitation of ^{13}C -, ^{15}N nuclei by

¹⁰**Safety note:** Use rotor caps made of zirconia or Vespel for experiments at low temperature. Standard Kel-F caps loosen at low temperature which may result in a rotor crash and damage to the radio frequency coil and stator in the MAS probe.

cross-polarization, best sensitivity at acceptable broadening of resonances was achieved at a set temperature of the instrument of 200 K.¹¹ Acceptable resolution of ¹³C-, ¹⁵N resonances is typically achieved at spinning frequencies of 8 kHz or higher.

- Specific settings of the NMR instrument depend on the experiment to be conducted as well as the equipment. The reader is referred to special literature as well as specifications provided by the manufacturer of the equipment (see Fig. 3 for an example). A common issue is the tolerance of equipment and samples to ¹H decoupling power. Resolution of ¹³C-, ¹⁵N resonances is best if 70 kHz of ¹H decoupling or higher is applied. Sample heating from decoupling is successfully mitigated by lowering sample temperature or by the use of low E-field probes.

9. CONCLUSIONS

The protocols described here allow preparation of several milligrams of stable isotope-labeled, functional cannabinoid receptor CB₂. The protein is solubilized in detergent micelles and purified with high yield by affinity chromatography. Subsequent reconstitution of CB₂ into POPC/POPS lipid bilayers ensures adequate stability of the receptor for structural studies by solid-state NMR spectroscopy at temperatures below ambient.

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Tandem affinity purification of CB₂ on Ni-NTA and StrepTactin resins

Fig. 1.

A, Schematic representation of the tandem affinity purification of the recombinant CB₂ protein. Fusion CB₂ protein is extracted from membranes in detergent micelles and purified by chromatography on Ni-NTA resin. The MBP expression partner is then removed by cleavage with TEV protease, and the released CB₂ is further purified by chromatography on StrepTactin resin. **B**, SDS-PAGE stained with Instant Blue. Lane 1, Molecular weight marker; 2, crude extract applied onto Ni-NTA resin; 3,4, elution fractions from Ni-NTA resin; 5,6, protein treated with TEV protease, applied onto StrepTactin; 7, flow-through fraction from StrepTactin; 8, wash fractions; 9,10, CB₂ protein eluted from StrepTactin resin.

Preparation of ^{13}C , ^{15}N -labeled CB_2 by *E. coli* fermentation

Fig. 2.

Schematic representation of the fermentation of *E. coli* BL21 (DE3) expressing CB_2 . The green trace (solid line) denotes the growth of the bacterial cell culture, blue trace (square dot) – glucose concentration in the medium; red trace (dash line) - ammonium concentration in the medium. Arrows indicate addition of glucose (blue square dot), ammonium (red round dot), IPTG- (solid yellow), and change of the temperature from 37°C to 20°C (thick green solid arrow).

Fig. 3.

Two-dimensional cross-polarization, dipolar assisted rotational resonance experiment (2-D ^{13}C - ^{13}C CP-DARR) (Takegoshi, Yano, Takeda, & Terao, 2001) on uniformly ^{13}C -, ^{15}N labeled CB₂ in proteoliposomes recorded at a temperature of 203 K and a sample spinning frequency of 10 kHz. The relatively high intensity of ^{13}C - ^{13}C cross-peaks in the spectrum confirms high, uniform enrichment of the protein with ^{13}C . The large number of resonances results in heavy superposition of signals. NMR parameters: ^1H preparation $\pi/2$ pulse=3.5 μs ; ^1H - ^{13}C cross-polarization at $(\gamma/2\pi)B_1$ =49 kHz for 1 ms, ^1H power during cross-polarization was linearly ramped from 100% to 80%; DARR ^{13}C $\pi/2$ pulse=4.5 μs ; DARR mixing time=100 ms. ^1H continuous wave decoupling at 10 kHz was applied during mixing. ^{13}C signals were acquired at 70 kHz ^1H dipolar decoupling using a spinal-64 decoupling sequence (Fung, Khitrin, & Ermolaev, 2000). Two-dimensional spectra were acquired with 1024 data points in the F_2 domain, 128 data points in the F_1 domain and 8,500 scans per F_1 increment.