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Preparation of stable isotope-labeled peripheral cannabinoid receptor CB2 by bacterial fermentation

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Abstract

We developed a bacterial fermentation protocol for production of a stable isotope-labeled cannabinoid receptor CB2 for subsequent structural studies of this protein by nuclear magnetic resonance spectroscopy. The human peripheral cannabinoid receptor was expressed in *Escherichia coli* as a fusion with maltose binding protein and two affinity tags. The fermentation was performed in defined media comprised of mineral salts, glucose and ¹⁵N₂-L-tryptophan to afford incorporation of the labeled amino acid into the protein. Medium, growth and expression conditions were optimized so that the fermentation process produced about 2 mg of purified, labeled CB2 per liter of culture medium. By performing a mass spectroscopic characterization of the purified CB2, we determined that one of the two ¹⁵N atoms in tryptophan was incorporated into the recombinant protein. NMR analysis of ¹⁵N chemical shifts strongly suggests that the ¹⁵N atoms are located in Trp-indole rings. Importantly, analysis of the peptides derived from the CNBr cleavage of the purified protein confirmed a minimum of 95% incorporation of the labeled tryptophan into the CB2 sequence. The labeled CB2, purified and reconstituted into liposomes at a protein-to-lipid molar ratio of 1:500, was functional as confirmed by activation of cognate G proteins in an *in vitro* coupled assay. To our knowledge, this is the first reported production of a biologically active, stable isotope-labeled G protein-coupled receptor by bacterial fermentation.

Keywords

Cannabinoid CB2 receptor; stable isotope-labeling; bacterial fermentation; G protein-coupled receptor

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Introduction

The peripheral cannabinoid receptor CB2 is a member of the large family of rhodopsin-like class A G protein-coupled receptors (GPCRs) that contains ~ 670 proteins [1–3]. These are highly hydrophobic membrane proteins comprised of seven trans-membrane α -helices [4–6] alternately connected by intra- and extracellular loops. CB2, primarily localized in the peripheral tissues, is involved in a large array of important physiological processes, in particular in immune regulation [3,7]. It has been demonstrated that specific ligands of CB2 affect various pathways involved in pain sensing [8], osteoporosis [9], atherosclerosis [9], and other diseases [7]. NMR studies may provide valuable insights into the structure and function of this receptor, paving the way for rational design of specific ligands.

A prerequisite for structural characterization of CB2 is the availability of milligram amounts of purified, functionally active protein. The expression levels of CB2 as well as of the majority of other GPCRs in natural tissues are quite low [10]; heterologous expression is currently the only technically feasible way to prepare these proteins in large quantities. Several heterologous hosts such as mammalian, insect, yeast, bacterial cells as well as cell-free expression have been tried with varied success for production of recombinant GPCRs for structural investigations. The production strategy is selected based on the yield, correct fold and homogeneity of the target receptor [11]. For example, the expression of human membrane proteins in mammalian cells has certain potential advantages, since this host possesses an adequate machinery to ensure correct folding and post-translational modifications of the recombinant GPCRs. At the same time, mammalian cells are usually characterized by low expression levels of the target protein, although in a few cases yields up to ~2 mg/L culture medium (adenosine A_{2A} receptor [12, 13] and β_2 -adrenergic receptor [10,13]) have been reported. In order to apply NMR techniques to structural studies of GPCRs, the expression strategy should be adapted to allow incorporation of stable isotopes into the target molecule for either uniform or site-specific labeling. Considering this important requirement, the use of a bacterial expression system offers more choices of labeling strategies and may be more cost efficient.

Expression in *Escherichia coli* (*E. coli*) cells [14] is characterized by high growth rates [13], significant biomass yields, and has a potential for producing homogenous (albeit non-glycosylated) [13,14] target proteins. *E. coli* cells can grow in a minimal medium of a defined composition which simplifies procedures for the incorporation of stable isotope-labeled precursors into the recombinant receptor [13]. We have previously reported preparation of functional CB2 in milligram quantities by cultivating *E. coli* cells in a complex medium [15]. By further adapting our strategy to fermentation in minimal medium, we explored the possibility of producing active CB2 receptor while trying to maximize the protein yield and minimize the use of expensive labeled nutrients.

While X-ray crystallography accounts for the vast majority of published structures of integral membrane proteins, its application to GPCRs is challenging, in large part due to the difficulties in obtaining well-diffracting crystals of these highly hydrophobic proteins in the presence of lipids and detergents [3,12,16]. Solid-state NMR spectroscopy potentially offers an important advantage by probing the structure of these membrane proteins in their natural environment, the lipid bilayer. The method requires incorporation of stable isotope labels, such as ¹⁵N- and/or ¹³C-labeled amino acids into the protein sequence, and the protein expression has to be performed in a well-defined medium. Here, we describe the development of a strategy to specifically introduce the ¹⁵N-labeled amino acid tryptophan into the CB2 sequence by bacterial fermentation in mineral salt medium (MSM) supplemented with glucose and a labeled amino acid. The labeled receptor was ligand-binding competent and activated G proteins in an *in vitro* coupled assay.

Materials and methods

Chemicals

$^{15}\text{N}_2$ -labeled L-tryptophan (98% isotope enrichment) was from Cambridge Isotopes (Cambridge, MA). All natural isotope-abundance amino acids and other chemicals were from Sigma-Aldrich (St. Louis, MO). Ligand CP55,940 was from Tocris (Ellsville, MO). [^{35}S]- γ -GTP was from Perkin Elmer (Waltham, MA). Lipids: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) sodium salt used for CB2 reconstitution were purchased from Avanti Polar Lipids (Alabaster, AL).

Expression strain and plasmid

Escherichia coli strain BL21 (DE3) was obtained from Stratagene (La Jolla, CA). Construction of plasmid pAY130 was described earlier [3].

Composition of the mineral salt medium (MSM), (modified on the basis of [17])

The MSM used in this study contained: 4.65 g/L Na_2SO_4 , 14.6 g/L K_2HPO_4 , 4.07 g/L $\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$, 1.2 g/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 1.66 mg/L $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 0.36 mg/L $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.2 mg/L $\text{MnSO}_4 \times \text{H}_2\text{O}$, 34.74 mg/L EDTA, 33.4 mg/L FeCl_3 , 0.128 mg/L $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 0.42 mg/L $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, 100 mg/L thiamine hydrochloride and 100 mg/L ampicillin. The concentration of NH_4Cl in flask experiments was 2.73 g/L. The concentration of NH_4Cl at the beginning of fermentation was 2.73 g/L, and was adjusted during the process as needed. The concentrations of glucose and citric acid varied, as indicated below.

Composition of amino acid media

We tested different concentrations of amino acids in minimal media (AAM) for their effects on the cell growth rate, levels of target protein production and functionality of CB2 in *E. coli* membranes. Amino acids were grouped according to their concentrations in the incubation medium as follows. Group 1: 4-hydroxyproline. Group 2: L-alanine, L-arginine monohydrochloride, L-asparagin anhydrous, L-aspartic acid, L-cysteine, L-cystine, L-glutamic acid, L-glutamine, glycine, L-isoleucine, L-leucine, L-lysine monohydrochloride, L-methionine, L-serine, L-threonine and L-valine. Group 3: L-histidine monohydrochloride monohydrate, L-phenylalanine, L-proline, L-tryptophan and L-tyrosin. The amino acid media contained all mineral salts, trace elements, vitamins and antibiotics as listed above. Additionally, ammonium chloride and glucose were provided at concentrations indicated in the text. Concentrations of amino acids are provided in Table 1.

Adaptation of BL21-130 to growth media

E. coli BL21 (DE3) cells harboring the plasmid pAY130 were cultivated in double strength YT medium supplemented with 100 mg/L ampicillin and 2 g/L glucose. The overnight grown cells were used to prepare 10% glycerol stocks and subsequently stored at -80°C . In order to perform cell adaptation, the glycerol stocks were used to inoculate the media of the desired composition. Cells grown in this first adaptation phase were used for preparation of yet another series of glycerol stocks. The procedure of successive cell growth and preparation of glycerol stocks was repeated two more times, thus allowing the culture to undergo a total of three media-adaptation stages. No further increase in the specific growth rates was observed after the third stage, and adaptation was considered to be completed.

Shake flask experiments

Shake flask experiments were performed in 125 mL flasks filled with 25 mL of medium. Incubation was performed on an orbital shaker at a speed of 230 rpm, and all cultures were

incubated in duplicate. Cells were cultivated at 37°C until the desired OD₆₀₀ was reached. The expression of the recombinant protein was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG), while the incubation temperature was simultaneously lowered over a period of 30 minutes to 20°C. Samples of cells for Western blot, ligand binding and G protein activation analysis were taken at various time points, as indicated.

Fermentation processes

Fermentation was carried out in a 3.0 L BioFlo® 110 Bench-Top Fermentor (New Brunswick, NJ) in a batch volume of 1 L. The cell cultivation was performed as batch process, and glucose and ammonium chloride were added in proportion of 4 to 1 (w/w) at the start of the process and in the course of fermentation, as needed. The concentration of dissolved oxygen with a set point of 30% was controlled by a cascade of stirring speed, flow of air, and the ratio of air and oxygen. Fermentation parameters were controlled and recorded by the NBS BioCommand® Plus software. A time profile contained a step of reduction of the fermentation temperature from 37°C to 20°C, 30 minutes prior to the addition of the inducer IPTG. The pH value was adjusted during the fermentation to 7.0 by the hardware-controlled addition of 10% NaOH and 10% H₃PO₄.

The typical experiment was performed as follows. Shake flasks containing 25 mL of MSM were inoculated with the glycerol stocks obtained from medium-adapted cells. The cells were grown to OD₆₀₀ = 1–1.5 and used to inoculate 300 mL of MSM in a 2L shake flask. The cells were grown overnight to the OD₆₀₀ = 3.0–3.5, collected by centrifugation, re-suspended in a small volume of tap water and injected into the fermentor, so that the OD₆₀₀ of the culture at the start of fermentation was approximately 1.0. Samples from the culture were taken every 30 minutes to monitor cell growth and concentration of glucose during fermentation. The Assure® 3 Blood Glucose Monitoring System (Arkray, USA) was used to determine the glucose content in the incubation medium. When the desired OD₆₀₀ was reached, the fermentation temperature was reduced to 20°C, and the CB2 protein production was induced with IPTG. The duration of induction varied between experiments from 30 min to 40 hours.

Preparation of ¹⁵N, ¹³C-uniformly labeled CB2

¹⁵N, ¹³C- uniformly labeled CB2 was prepared by fermentation of *E. coli* BL21-21(DE3) harboring plasmid pAY130 using the procedure described in the section “Fermentation processes” with the following modifications. ¹⁵NH₄Cl₂ (99% enrichment; Cambridge Isotope Laboratories, Andover, MA) was used as the sole source of nitrogen, and D-glucose (U-13C₆, 99%; Cambridge Isotope Laboratories, Andover, MA) - as the sole source of carbon. Concentration of glucose in the medium at the start of fermentation was 10 g/L, and concentration of the ammonium chloride – 2.74 g/L. Concentration of glucose was monitored at frequent time intervals, and its content in the medium adjusted as needed by injecting additional portions of the glucose/ammonium chloride stock solution (at a 4:1 w/w ratio), so that the concentration of glucose in fermentation medium never fell below 3 g/L. Induction of the CB2 protein production was started when OD₆₀₀ = 10 of cell culture was reached, and cells harvested 10 hours post-induction. Purification of the uniformly labeled ¹⁵N, ¹³C- CB2 protein and its reconstitution into liposomes was performed by following the protocols described below for purification and lipid-reconstitution of the ¹⁵N-Trp-labeled CB2. The details of preparation and characterization of ¹⁵N, ¹³C-CB2 will be described elsewhere (K. Vukoti et al, in preparation).

Analysis of CB2 protein content by Western blot

Cell samples collected during the fermentation were normalized by their optical density. The cells were then centrifuged for 2 min at 14,000 rpm in a table-top centrifuge, and the cell pellet was re-suspended in SDS sample buffer (BioRad, Hercules, CA) and briefly sonicated. The

proteins were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and probed with anti-HIS INDIA-HRP reagent (Pierce, Rockford, IL).

To analyze the CB2 content in membranes, samples were normalized by their total protein concentration. Proteins were resolved in SDS-PAGE, and CB2 levels determined as described above.

Membrane preparation and determination of the protein concentration

The crude membrane fraction was prepared as follows. All procedures were performed at +4°C or on ice. Cells were collected by centrifugation at 20,000×g for 10 min, washed once with cold PBS buffer, centrifuged again, and resuspended in a small volume (3–5 ml) of cold PBS buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by passing the cell paste twice through a French Press (Thermo Fisher, Pittsburgh, PA). Unbroken cells and cell debris were removed by centrifugation at 20,000×g for 10 minutes, and the supernatant was subjected to high-speed centrifugation (170,000×g, 1h). The pellet was re-suspended in PBS buffer supplemented with protease inhibitors, and centrifuged again. The resulting membrane pellet was re-suspended in a small volume of PBS buffer supplemented with protease inhibitor cocktail and 10% (w/v) sucrose, aliquoted, flash-frozen in liquid nitrogen, and stored at –80°C until use. The protein concentration in membrane preparations was determined by a DC protein assay kit (BioRad, Hercules, CA).

Functional characterization of CB2 receptor

The functional activity of CB2 in membranes and in proteoliposomes was routinely assessed by performing a G protein activation assay as described previously [15] using purified Gα_{i1} and Gβ₁γ₂ proteins and [³⁵S-γ-GTP] (Perkin Elmer, Waltham, MA).

Levels of functional receptor in membrane samples were also analyzed by a ligand saturation binding assay as described previously [15].

Purification of recombinant CB2

CB2-130 fusion protein was extracted from the biomass with a mixture of detergents: dodecylmaltoside (DM, 1%, w/v), CHAPS (0.5% w/v) supplemented with cholesteryl hemisuccinate (CHS, 0.1%, w/v). This combination of detergents was chosen as the most efficient one for solubilization of the recombinant receptor and for preservation of its activity based on earlier work by R. Grisshammer on the recombinant neurotensin receptor [18] and our preliminary studies of stability of the CB2 receptor in detergent micelles of different composition (A. Yeliseev et al., in preparation). Fusion protein CB2-130 was purified by affinity chromatography on Ni-NTA Sepharose, digested with TEV protease and the CB2 cleavage product further purified by chromatography on a StrepTactin Macrorep column following the procedure described earlier for proteolytic cleavage and purification of CB2-125 [3]. Purified CB2 was concentrated in a centrifugal spin concentrator (Orbital Biosciences, Topsfield, MA) with a 30 kDa molecular mass cut off, and the protein concentration determined with a Bio-Rad DC kit. This final step of protein sample preparation results in a co-concentration of some of the components of the elution buffer. Typically, the 40 μM CB2 preparation contains DM at a concentration of 0.4–0.6% (w/v), CHAPS - 1.5–2%, (w/v), CHS – 0.4–0.6% (w/v) (T. Kimura et al, in preparation).

Reconstitution of the purified CB2 into liposomes for functional assay

Reconstitution of the purified CB2 protein into liposomes was performed using detergent-removing resin (Thermo Scientific, Waltham, MA). A bolus of 100 μg of the purified protein

at a concentration of 1.2 mg/mL in a buffer containing DM/CHAPS/CHS micelles was mixed with 1 mg of a mixture of the lipids POPC/POPS (4/1, mol/mol) dissolved at a concentration of 5 mg/ml in 1% aqueous solution of the zwitterionic detergent lauryl dimethylamine N-oxide (LDAO). Alexa Fluor 488 fluorophore-labeled CB2 (2 μ g) were added to the preparation in order to quantify the recovery of protein in proteoliposomes. The solution of POPC/POPS also contained 0.1 μ g of fluorophore-labeled lipid, DilC18(5) (Invitrogen, CA) added for quantification of lipid recovery. The combined protein-lipid-detergent solution was adjusted to a final volume of 315 μ L, and 300 μ L of the solution loaded onto a 1.5 mL column of Detergent Removing resin (Thermo Scientific, Waltham, MA); the remaining 15 μ L were used to prepare the calibration curves for CB2- and lipid quantification. The liposomes were eluted with PBS buffer and collected in a total volume of 800 μ L. CB2 and lipids were quantified by fluorescence from AlexaFluor488-labeled CB2 and DilC18(5), respectively. Measurements were performed on a Synergy HT Microplate reader (BioTek, Winooski, Vermont). AlexaFluor was excited at 488 nm and emission detected at 528 nm. DilC18(5) was excited at 590 nm and emission detected at 645 nm. Cross-talk between the fluorophores was negligible at these settings.

Determination of the labeling efficiency for labeled L-tryptophan in CB2

The CB2 tryptic peptides were prepared following the protocol for preparation of peptides of the recombinant neurotensin receptor outlined in [19]. Briefly, 50 μ L of CB2 solution at a concentration of 1.5 mg/mL in detergent buffer (50 mM Tris-HCl, pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, supplemented with CHAPS, CHS and dodecylmaltoside at the concentrations indicated above) were mixed with methanol (150 μ L) and chloroform (50 μ L). A solution of 10% trifluoroacetic acid (aqueous) (100 μ L) was then added and mixed. The two phases were separated by centrifugation (8,944 $\times$ g at 10°C, for 20 min), yielding precipitated protein at the interface. The bulk of the upper and lower phases were removed without disrupting the precipitated protein at the interface. Methanol (150 μ L) was added to the precipitate and mixed. The protein pellet was recovered by centrifugation, after which the majority of the methanol was removed with a pipette tip. The protein pellet was then allowed to dry.

For CNBr cleavage, the dried protein pellet was dissolved in 90% aqueous formic acid (100 μ L). CNBr (5 μ L) was added to the dissolved receptor and mixed for 2 hours at room temperature in the dark, after which CNBr was removed using a vacuum centrifuge. Peptides were acidified with 0.5% TFA (500 μ L) and left to stand overnight at room temperature. TFA was removed using a vacuum centrifuge and peptides were washed with water (2 $\times$ 100 μ L) with drying in between each wash step. The peptides generated by the CNBr treatment were solubilized in 0.2% RapiGest (25 μ L final volume) (Waters, Milford, MA) before performing reduction and alkylation of cysteines. Reduction of disulfide bonds was performed by the addition of 45 mM DTT (2.5 μ L) to the sample and incubation at 55°C for 30 minutes. For the subsequent alkylation of free cysteine residues, 100 mM iodoacetamide (2.5 μ L) was added and incubated at room temperature in the dark for 30 minutes. The CNBr generated, cysteine alkylated peptides were further digested with trypsin and/or chymotrypsin in the presence of 0.2% RapiGest. A 1:5 enzyme-to-protein ratio was used. Digestion was carried out at 37°C overnight. After digestion, RapiGest was hydrolyzed by the addition of formic acid to give a final concentration of 30% acid. The peptide mixture was incubated at 37°C for 30 minutes and then centrifuged for 5 minutes. The supernatant was transferred to a clean vial and stored at -20°C until required for analysis.

An aliquot of the CB2 digest mixture (10 μ L) was loaded onto a trap cartridge packed with C8 stationary phase (Michrom Bioresources) for desalting using 0.2% aqueous formic acid (mobile phase A). Peptides were subsequently separated on a 15 cm $\times$ 75 μ m ID column with an integrated spray tip, packed with C18 stationary phase (Michrom Bioresources), using an

increasing proportion of acetonitrile containing 0.2% formic acid (mobile phase B). The eluent from the column was directly electrosprayed into the mass spectrometer. Reversed phase LC was performed using a UPLC system (CVC MicroTech) and tandem mass spectrometric analysis was performed using an LTQ-Orbitrap mass spectrometer (ThermoFisher, Bremen, Germany). Data were acquired in data-dependent mode using Xcalibur software (ThermoFisher). In each cycle, the five most intense ions detected in the full scan MS of the Orbitrap mass analyzer were automatically isolated and fragmented in the linear ion trap. Bioworks (ThermoFisher) was used to generate data files and a Perl script was used to generate a Mascot Generic Format (MGF) file that was then used to search the Swissprot protein database using the MASCOT (Matrix Science, London, UK) search engine. Typical search parameters include: 5 ppm error for MS mode and 0.5 Da for MS/MS mode. Peptide identifications were verified by manual interpretation of MS/MS spectra.

¹⁵N-MAS NMR measurements

About 1 mg of the ¹⁵N-Trp-labeled purified CB2 were reconstituted into POPC/POPS bilayers at a protein/lipid molar ratio of 1/500 as described above. Proteoliposomes were pelleted by ultracentrifugation at 500,000×g for 12 hours and the pellet transferred to a 4-mm MAS rotor with a Kel-F insert that restricts sample volume to 50 µL (Bruker BioSpin, Billerica, MA). The ¹⁵N-CP-MAS NMR spectra were recorded on AV800 NMR spectrometer equipped with a 4-mm 1H/13C/15N-CP-MAS variable temperature probehead (both Bruker BioSpin, Billerica, MA). The instrument settings were as follows: 10 kHz MAS spinning frequency, −18°C sample temperature, 800.18 MHz ¹H- and 81.08 MHz ¹⁵N-resonance frequencies, a 3.7-µs 90° ¹H pulse, 1-ms ¹H-¹⁵N-cross-polarization at 50 kHz with an 100-80% linear ramp on the ¹H channel, 60 kHz ¹H decoupling during ¹⁵N detection using a spinal-64 sequence with a pulse length of 10 µs, 56,600 scans, 1024 data points, 5-µs dwell time, 2-s delay time between scans.

For comparison, the ¹⁵N spectrum of a 2-mg sample of homogeneously ¹⁵N-, ¹³C-labeled CB2, reconstituted under identical conditions, and recorded with identical spectrometer settings but 29,500 scans was acquired. The ¹⁵N-chemical shift scale was calibrated by setting the midpoint of the intense ¹⁵N-amide resonance to 120 ppm.

Results

Production of milligram quantities of functional GPCRs in isotope-labeled form in a heterologous system presents unique technical challenges. In our studies we focused on developing an efficient protocol for expression of functional CB2 receptor in *E. coli* cells cultivated in a minimal medium. We used the CB2-130 fusion construct comprised of the CB2 protein flanked by the maltose binding protein (MBP), a TEV-protease recognition site followed by a Strep-tag (at the N-terminus), and a decahistidine tag at the C-terminus, Fig. 1. The advantages of the fusion protein for production of functional CB2 by *E. coli* fermentation were previously discussed [3].

Selection of media and initial optimization of culture conditions in shake flasks

Adaptation of BL21 (DE3)/pAY130 to growth media—To develop minimal media that supported fast cell growth and accumulation of large biomass, we initially performed small scale experiments in shake flasks. Preliminary experiments were performed in order to find optimal conditions of preparation of a bacterial pre-culture for fermentation in minimal medium. *Escherichia coli* BL21 (DE3)/pAY130 cells pre-grown in 2xYT medium supplemented with 0.2% glucose were further adapted to minimal media according to the protocol described in Materials and Methods. The efficiency of the cell adaptation was assessed

by calculating the specific growth rate, doubling time, and yield coefficient of biomass to substrate (Table 2).

The initial cell growth rate in M9 medium was higher than in minimal salt medium (MSM). However, the biomass yield in M9 medium was only one half of that of MSM. Since the concentration of the carbon source (glucose) was 1% in both types of media, we concluded that MSM medium was better suited for subsequent expression experiments since sufficiently high biomass yield is an important prerequisite for development of an efficient labeling strategy.

A large number of samples generated in these experiments were analyzed for the expression levels and activity of the recombinant receptor. Routine assessment of the expression levels of the recombinant CB2 was performed by semi-quantitative Western blot, while analysis of the functional activity of CB2 receptor was performed in an *in vitro* coupled G protein activation assay, as described in Materials and Methods. The advantages of this assay, compared to saturation ligand binding are its ability to process large number of samples, a better signal-to-noise ratio, and good reproducibility. An additional ligand saturation binding assay was performed on select samples.

We then proceeded with supplementation of the medium using various sources of carbon and nitrogen in order to study requirements for accumulation of high levels of functional receptor. Cells undergoing adaptation to a medium containing amino acids as sole source of carbon and nitrogen showed no increase in growth rate during the second adaptation phase. The addition of 2g/L of glucose did not noticeably affect the growth rate (Table 2). However, the accumulation of biomass with addition of glucose was significantly higher than in M9 medium suggesting that supplementation of MSM with amino acids and glucose can potentially be considered for incorporation of isotope-labeled amino acids into the CB2 protein.

The specific growth rate of BL21-130 cells did not increase after the third adaptation phase in MSM supplemented with glucose and ammonium chloride (Table 3). Thus, the cells collected after three adaptation steps were used in all subsequent experiments.

After these basic parameters of media composition were determined, we further studied the effects of density of cell culture and duration induction on (i) expression levels, (ii) temperature, and (iii) supplementation with amino acid(s).

Effect of cell density at induction on protein yields and functionality—We further investigated the effect of cell density at the time of induction on levels and functional activity of the recombinant CB2 protein in *E. coli* membranes. The experiments were carried out in 125 mL shake flasks containing 25 mL MSM supplemented with 5 g/L glucose. The protein expression was induced at optical densities of the culture of 0.5, 1.0 and 3.0 with 0.5 mM IPTG, and the incubation temperature was immediately changed to 20°C. Duplicate samples were used for every condition tested. At 29 hours post-induction 5 g/L glucose was added to one of the two duplicate samples. The glucose addition resulted in further increase of biomass of the samples induced at optical densities of 0.5 and 1.0 (Fig. 2A), while no increase in biomass accumulation was observed for the cells induced at OD₆₀₀= 3.0 (data not shown). All cells were incubated until there was no further increase in optical density. At this point cell samples were taken and protein expression levels and activity analyzed.

The Western blot analysis of CB2 protein levels indicated an increase in CB2 content in the cells induced at high optical density (Fig. 2B). Higher glucose concentration (10 g/L instead of 5 g/L) did not result in an increase of CB2 levels in the membranes.

The cost of the labeled precursors used to produce stable isotope-labeled proteins can be significantly reduced when the cells are pre-grown in a medium containing nutrients at natural abundance and isotopically labeled compounds are supplied only at the later stage of growth and during protein expression [20]. Therefore, we studied whether a higher OD₆₀₀ at induction would result in a further increase of CB2 levels in *E. coli* membranes. Recombinant protein production was induced in cells re-suspended in MSM to optical densities of 4.5, 5.5, 6.0, and 12.0. The comparison of the CB2 expression levels was performed by Western Blot analysis. As a reference, we used BL21-130 cells cultivated in double strength YT medium and induced at OD₆₀₀=0.6. The results of these measurements are presented in Fig. 3. The CB2 concentration increased with an increase in cell optical densities at induction and reached a maximum at the induction OD₆₀₀ of 10. The specific activity of the CB2 receptors increased gradually with optical densities at induction, reaching a maximum at OD₆₀₀ = 4.5 (results not shown).

Influence of the temperature of incubation on yield and the functionality of CB2

—The choice of temperature of cultivation is important for functional expression of membrane proteins, including recombinant GPCRs, because it affects the rates of protein production. A misalignment of the rates of protein synthesis with the capacity of cells for proper folding and insertion into membranes commonly results in target protein misfolding and formation of insoluble inclusion bodies. We have demonstrated earlier that functional CB2 receptor is successfully produced in *E. coli* BL21 cells cultivated in 2xYT medium at 20°C. In order to determine conditions for high-level production of the correctly folded CB2 protein in minimal medium, the following experiment was performed. Cells were grown in MSM supplemented with 0.5% glucose to OD₆₀₀ = 1, after which point the temperature was changed to either 16°C, 20°C or 27°C, and protein production was induced by the addition of 0.5mM IPTG. Cells were collected at 40 hours post-induction, and the levels of CB2 in membranes and functional activity of the recombinant receptor determined. The specific activity of CB2 reaches a maximum at a temperature of 20°C after induction, and decreases slightly at 27°C (Fig. 4). The levels of CB2 protein in membranes increased steadily with the increase of the cultivation temperature above 20°C. Therefore, we concluded that an increase of the induction temperature to above 20°C results in a significant accumulation of non-functional receptor.

Expression of active CB2 in media supplemented with amino acids—Our preliminary studies showed that while the difference in concentrations of amino acids in AAM1, AAM2 and AAM3 did not result in changes of growth rates, cultivation in AAM1 yielded lower amounts of biomass (data not shown). Since the yield of biomass is an important parameter that ultimately affects the total yield of the receptor in fermentation, we performed cultivation in AAM2 and AAM3 by supplementing these media with ammonium chloride and glucose. Additionally, AAM3 was supplemented with unlabeled L-tryptophan at the time of induction. MSM supplemented with glucose was used as a control in these experiments. The cell cultivation was performed in 400 mL of medium, and the cell samples for Western blot and activity tests were taken at 0 h, 4 h, 10 h, 18 h, 28 h and 42 h after induction. The protein production was induced at an optical density of 1.0 followed by an immediate switch to the incubation temperature of 20°C (Fig. 5).

An increase in specific growth rates of BL21-130 and a higher yield of biomass were observed when glucose was added to the amino acid medium. The addition of ammonium chloride at concentrations of 2.73 g/L to the AAM2 medium slightly reduced the growth rates, and the addition of tryptophan had no significant effect on growth (Fig. 5A). The expression levels and functionality of produced CB2 were analyzed by Western blotting and a G protein activation assay, respectively (Fig. 5, B). Activity of the receptor was also confirmed by the saturation ligand binding assay (data not shown).

Levels of functional receptor expressed in AAM reached a maximum early in the course of fermentation, and decreased towards the end of fermentation. This contrasts the results obtained from cultivation in MSM supplemented with glucose as a sole carbon source, in which the specific activity of the receptor was almost constant through the entire duration of fermentation. Maximal specific activity of the recombinant CB2 produced in AAM media was achieved 4 hours after induction. On the one hand, the addition of glucose to the AAM media did not substantially affect the activity profile. On the other hand, glucose used as a sole carbon source resulted in the highest yield of functional receptors per unit of biomass. The levels of active CB2 in MSM-glucose medium were at least 75% of that of the receptors produced in complex 2xYT medium. The content of CB2 protein in *E. coli* membranes correlated well with activity (data not shown).

Subsequent optimization of fermentation conditions was performed in the BioFlo 110 fermentor, focusing on maximizing yield of functional CB2.

Fermentation of *E. coli* for production of functional CB2

Optimization of the expression of CB2 in fermentation processes—To optimize the yield of active receptors in batch fermentation we performed two types of fermentation trials. In the first, bacterial cultivation was performed in MSM supplemented with glucose and ammonium chloride as sole sources of carbon and nitrogen, respectively. When the cell culture reached an optical density of 10, the fermentation temperature was reduced from 37°C to 20°C within 30 minutes, and the protein expression was induced with 0.5 mM IPTG. Induction of protein synthesis was performed at an OD₆₀₀ of 10 of cell culture based on the results of test fermentation runs (results not presented), so that the projected accumulation of the recombinant receptor in the biomass is in the range of 1–2 mg in 1 L of culture. Cell samples were taken at different time points after induction, and analyzed as described above. The second fermentation was performed essentially in the same way as the first one, with the exception that 200 mg/L of L-tryptophan (unlabeled) was added at induction point. When glucose was used as sole carbon source, the levels of functional CB2 protein reached a maximum at 10 hours after induction. In contrast, the addition of tryptophan at the time of induction resulted in a shift of the maximum of CB2 activity to 4 hours post-induction (Fig. 6A, B). The levels of expression of functional CB2 receptor in *E. coli* membranes were monitored by measuring rates of G protein activation (Fig 6A). Results were confirmed by a ligand saturation-binding assay with ³H-CP55940 on membranes from (i) control cells grown in 2xYT medium and collected 40 hours post-induction; (ii) cells grown in MSM supplemented with glucose and ammonium chloride and collected 10 hours post-induction; (iii) cells grown in MSM supplemented with glucose, ammonium chloride and L-tryptophan, collected 4 hours post-induction. This result correlates well with the previously described effect of supplementation of MSM with a mixture of amino acids (Fig. 5B) where maximal levels of accumulation of functional receptor were observed early in fermentation. Thus, in order to maximize yield of the active protein, in experiments with labeled ¹⁵N₂-tryptophan, biomass was harvested 4 hours post-induction.

Production, purification and characterization of ¹⁵N-Trp-labeled CB2

Fermentation with labeled ¹⁵N₂-L- tryptophan—The above results helped us to design a strategy for expression of functional CB2 protein labeled with ¹⁵N₂-L-tryptophan. The same fermentation conditions may be applied for incorporation of ¹³C-labeled tryptophan, an amino acid which is significantly more expensive and therefore hard to use for optimizing expression protocols. We have been aware that the ¹⁵N label at the amino group of tryptophan could be lost due to trans-amination in *E. coli* [21,22]. However, this is of no consequences for the applicability of the procedure for production of ¹³C-labeled CB2. [21,23–25].

The cells were grown at 37°C to OD₆₀₀ ~ 20, at which point the fermentation temperature was reduced to 20°C, and ¹⁵N₂-labeled L-tryptophan was added to the medium. As we determined earlier (Fig. 6), addition of tryptophan to the MSM supplemented with glucose and ammonium results in accumulation of maximal amounts of active CB2 receptor early in fermentation at 4 hours after addition of IPTG. Therefore, the protein production was induced 2.5 hours after addition of labeled L-tryptophan, by supplementing the medium with 1.0 mM IPTG, and cells were harvested 4 hours after induction (Fig. 7A). The levels of expressed CB2 as analyzed by Western blot increased steadily during fermentation, up to the harvest point four hours post-induction (not shown). The levels of functional receptor as assessed by the G protein activation progressively increased and paralleled the increase of biomass during the course of fermentation, up to the harvest point (Fig. 7B).

Thus, the optimized fermentation process led to the successful expression of CB2 receptor in MSM supplemented with a stable isotope-labeled amino acid. The levels of recombinant receptors reached 87% of that in a reference sample (produced in a shaker culture cultivated in 2x YT medium) [15].

Purification and reconstitution of labeled CB2 into liposomes—The purification of the ¹⁵N-Trp-labeled CB2 was performed essentially as described previously [3]. A total of 1.8 mg of the purified CB2 protein was isolated from the biomass collected from 900 mL of cell culture (Fig 8, A, B). To determine functional activity of CB2, one hundred micrograms of the purified protein was reconstituted into a lipid matrix consisting of POPC/POPS (8/2, mol/mol) at a protein-to-lipid ratio of 1:500 (mol/mol), using the protocol described in the Materials and Methods. The mixture of detergents used to prepare the protein sample contained CHS; this lipid-like compound is not removed by the detergent-absorbing resin, and almost entirely incorporates into the lipid matrix upon receptor reconstitution. Consequently, the resulting proteoliposomes contain POPC/POPS/CHS, approximately at a ratio 6/2/2 (mol/mol/mol).

Functional characterization of CB2 in liposomes—While a ligand saturation binding assay is commonly used to quantify the receptor binding sites, its application to analysis of CB2-proteoliposomes is difficult due to the strong partitioning of the hydrophobic ligand (³H-CP55940) into the lipid matrix. Another difficulty is that unlabeled ligand CP55940 is added for stabilization of CB2 receptor during purification (see Materials and Methods), and significant quantities of it are incorporated into liposomes, along with the receptor. Typically, about 2-fold molar excess of CP55,940 (over the protein) is found in CB2-proteoliposomes (Vukoti et al, in preparation). Instead, we routinely analyze CB2-proteoliposome samples by performing a G protein activation assay on the receptor by adding the purified subunits of Gα_{i1}, Gβ_{1γ2} proteins. By comparing the rates of activation of G proteins on CB2-proteoliposomes with that of a standard sample, we can more accurately determine the content of active protein. Importantly, this test reports on the physiologically relevant functional activity of the receptor that is (arguably) more informative than ligand binding.

By performing the G protein activation assay on proteoliposomes (Fig 8, C), we estimate that between 50 and 100% of the reconstituted receptor retained functionality.

Mass spectrometry analysis of the ¹⁵N-labeled CB2 protein—Mass spectrometric analysis of the mixture of peptides generated from CNBr- followed by trypsin digestion resulted in observation of 70% sequence coverage of the CB2 receptor. Figure 9 shows the sequence of CB2-130 and observed peptides are highlighted. Labeled tryptophan containing peptides can be distinguished from their unlabeled counterparts in the mass spectrometer by their increased molecular weight, i.e.; 2 Da (Δ2 for M+H, Δ1 for M+2H). Comparison of the relative intensities of heavy/light pairs of peptides therefore allows assessment of the efficiency of metabolic labeling of CB2 [26,27].

MS spectra were interrogated for light/heavy pairs. The spectral pattern of the heavier, labeled peptides were calculated from spectral patterns of the light, unlabeled peptides using the expected mass shift, with consideration of the state of ionization. MS and MS/MS spectra for heavy/light pairs for tryptophan containing peptides SGSWSHPQFEK (+2) and ITPWPDSR (+2) are shown in Fig. 10. Incorporation of labeled tryptophan with two ^{15}N atoms (amide, and indole ring) for the two peptides (as well as for other identified tryptophan-containing peptides from CB2, data not shown) was not observed, whereas peptides with one ^{15}N -atom at tryptophan were observed at high signal intensities. MS/MS spectra of the heavy peptides confirmed the peptide sequence and a content of one ^{15}N atom per tryptophan residue (data not shown). More importantly, the results confirm a minimum of 95% efficiency of incorporation of the labeled ^{15}N -Trp into the CB2 sequence (Fig. 10).

^{15}N -NMR experiments—The ^1H - ^{15}N -CP-MAS NMR spectra of ^{15}N -Trp-CB2 and, for comparison, of uniformly labeled ^{15}N -, ^{13}C -CB2 are shown in Fig. 11. The spectrum of ^{15}N -Trp-CB2 shows a single ^{15}N -resonance with a chemical shift of 130 ppm. It has a frequency offset of about 10 ppm from the midpoint of the ^{15}N -amide resonance band of the uniformly ^{15}N -, ^{13}C -labeled CB2. The detection of a single resonance for ^{15}N -Trp-CB2 indicates that only one ^{15}N nucleus per tryptophan was retained. The chemical shift identifies the labeled site as the ^{15}N - ϵ -resonance of the indole ring [28]

Discussion

Expression of GPCR with labeled amino acid(s) can provide insights into the mechanisms of ligand-receptor interactions. For example, it has been suggested that tryptophan residues located in transmembrane helices participate in ligand recognition and binding in several GPCRs [29,30]. Thus, the labeled tryptophan could be used to probe receptor-ligand interaction, and to gain insight into the structure of the ligand-binding pocket. It is important to note that the biosynthetic pathway for tryptophan is a very energy-consuming process [31], and *E. coli* cells possess a system for active transport of tryptophan into the cell [32]. Therefore, the uptake of tryptophan from the culture medium, as opposed to its *de novo* biosynthesis, is likely to be the major source of this amino acid during the induction.

The goal of this study was to develop fermentation in minimal medium for efficient incorporation of stable isotope-labeled amino acid(s) into the peripheral cannabinoid receptor CB2. While we have earlier reported synthesis of functional CB2 in *E. coli* fermented in rich medium [3,15], production of this integral membrane receptor in *E. coli* cultivated in minimal medium proved to be challenging. Of critical importance was to test feasibility of fermentation in minimal medium for production of recombinant CB2 at high yield. Furthermore, fermentation conditions in MSM had to be optimized to ensure expression of functional protein. Lastly, fermentation had to be optimized to minimize the use of the expensive, labeled tryptophan while maintaining a high level of CB2 labeling.

Our initial studies focused on adaptation of the bacterial culture to the minimal medium. The three-step medium adaptation of the BL21 (DE3) cells harboring the plasmid pAY-130 resulted in a maximum specific cell growth rate of 0.53 g/(g*h) in media containing glucose as sole carbon source. The addition of citric acid to this medium did not alter the cell growth rate and biomass accumulation significantly. The use of MSM supplemented with amino acids as sole sources of carbon and nitrogen led to high specific growth rates but low levels of expression of the recombinant receptor.

There was a strong dependency of the expression levels of CB2 on the optical density of the cell suspension at the point of induction of recombinant protein synthesis. We observed a positive correlation between the concentration of cells at the time of induction and levels of

accumulated CB2 at the end of fermentation. At the same time, the specific functional activity of the recombinant receptor did not change significantly with the increase of biomass concentration at the time of induction. This contrasts observations made for the human adenosine A_{2a} receptor produced in yeast *Saccharomyces cerevisiae* [5] where yield of the receptor did not increase with the OD of the cell culture at the time of induction.

We determined earlier that the correct folding and functionality of the recombinant CB2 produced by fermentation of *E. coli* cells in rich 2xYT medium is strongly affected by the temperature of induction. Incubation at 20°C was found to be suitable for production of functional receptor [15]. We further proceeded to investigate the effects of the incubation temperature on expression levels and functional activity of CB2 produced by fermentation in MSM. While the total amount of recombinant receptor increased linearly with an increase of induction temperature from 16 to 27°C, fermentation at 20°C resulted in production of the highest amounts of functional CB2. Thus, it appears that incubation at 20°C is optimal for correct folding and efficient insertion of the recombinant CB2 into cell membranes. Our observations correlate with observations for expression of other GPCRs in *E. coli* and in yeast, where lower induction temperatures resulted in increased yield of functional receptors as well [5,33].

We studied whether supplementation of the MSM with amino acid(s) as single source of carbon and nitrogen can be used for production of the recombinant GPCR with specific labeled amino acids. This strategy was used successfully for production of other stable isotope-labeled proteins for NMR structural studies [20,34,35]. While it is possible to produce functional recombinant CB2 by fermentation of BL21-130 in minimal medium supplemented with amino acids as sole source of carbon, the yield of biomass and the level of the CB2 protein were quite low. Therefore, considering the high cost of fermentation in a medium with stable isotope-labeled amino acids, this approach is not practical. Thus, we performed further optimization of the fermentation conditions by supplementing the growth medium with glucose (carbon source) and ammonium chloride (nitrogen source). In this experiment, the amino acid (tryptophan) at natural isotope abundance was added to the medium shortly prior to the induction of expression of recombinant protein. We observed that addition of tryptophan results in accumulation of maximal levels of functional CB2 early in fermentation, just after four hours after induction. This contrasts results obtained with MSM supplemented with glucose as a sole carbon source, where maximum CB2 accumulation is reached 10 hours post-induction.

Based on these observations, we further modified our expression strategy as follows. We supplemented the defined MSM with glucose to produce a high biomass concentration prior to induction. To minimize the use of ¹⁵N₂-labeled L-tryptophan, this amino acid was added to the medium 2.5 hours before addition of IPTG. This time period was chosen arbitrarily, to allow *E. coli* cells to take up the amino acid from the medium and to adjust their metabolism accordingly. Functional activity of the CB2 protein isolated from the biomass was confirmed by the G protein-activation assay.

To study the incorporation of labeled tryptophan into the CB2 sequence, the purified receptor was cleaved with CNBr, followed by trypsin digestion, and its peptides were analyzed by LC-MS/MS. The efficiency of heavy tryptophan incorporation was assessed by comparing the relative intensities of the light/heavy pairs of selected peptides [27]. Results show the incorporation of more than 95% of heavy tryptophan with one ¹⁵N atom per tryptophan residue. By solid-state NMR we identified the ¹⁵N-labeled site as the nitrogen atom of the indole ring of tryptophan. While we do not know the exact mechanism by which one of the ¹⁵N atoms was lost from the tryptophan molecule during fermentation, it is likely that the amino group of tryptophan was replaced by transamination through action of the aromatic amino acid aminotransferase of *E. coli* [21,22,24,25].

In summary, we have developed a protocol for production of milligram quantities of stable, isotope-labeled cannabinoid receptor CB2 by fermentation of *E. coli* in minimal medium. Important features of this procedure are (i) fermentation of bacterial cells in a medium supplemented with glucose to ensure sufficient accumulation of biomass and high yield of the target protein and (ii) addition of the labeled amino acid shortly prior to induction to minimize the use of expensive labeled materials. The CB2 protein was isolated and reconstituted into liposomes in a functionally active form, and the yield of the purified protein was ~2 mg from 1 L of cell culture. Thus, the feasibility of production of the cannabinoid receptor CB2 containing labeled tryptophan for NMR structural studies has been demonstrated. To our knowledge, this is the first reported production of a full-length, biologically active, stable isotope-labeled G protein-coupled receptor by bacterial fermentation.

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Fig 1.
Schematic representation of the CB2-130 fusion protein

Fig. 2.

A. Growth curves of BL21/pAY130 cells induced at different cell densities.

B. Western blot analysis of CB2 accumulation in cells induced at different cell densities. I_{0.5}, I₁, I₃, samples collected at 20 hours post-induction from cultures induced at OD₆₀₀=0.5, 1.0 and 3.0, respectively. F_{0.5}, F₁, F₃, samples collected 40 hours post-induction from cultures induced at OD₆₀₀=0.5, 1.0 and 3.0, respectively. C₂₀ and C₄₀, negative control cells (without addition of IPTG) collected at 20 h and 40 h time points. M, molecular weight standards. The incubation medium contained either 5 g/L or 10 g/L glucose as indicated.

Fig. 3.

Levels of CB2 in *E. coli* membranes depend on the optical density of cells at the time of induction. Expression experiments were performed in MSM medium. Levels of CB2 protein expressed in 2xYT medium are taken as 1.0.

Fig. 4.

Expression levels and specific activity of recombinant CB2 in *E. coli* as function of incubation temperature. The levels and activity of CB2 are presented relative to levels and activity in samples expressed in 2xYT medium. The levels of CB2 protein in membranes were quantified by Western blot, and activity – by a G protein activation assay.

Fig. 5.

A. Growth of BL21 (DE3) pAY130 in amino acid/MSM media. The induction was started at an optical density of 1.0. 400 mg/L L-tryptophan was added at induction to the cells cultivated in AAM 3.

B. G-protein activation assay of CB2 expressed in *E. coli* cells cultivated in MSM supplemented with amino acids. Cell samples were taken 4 h, 10 h, 18 h, 28 h and 42 h post-induction. Control: CB2 expressed in *E. coli* BL21-130 cells cultivated in 2xYT.

Fig. 6. Functional activity of CB2-130 in *E. coli* membranes grown in various media

A. G protein activation on membranes. Cells were cultivated in MSM containing (i) glucose or (ii) glucose and L-tryptophan as carbon source. The functionality of CB2 protein at different time points of the fermentation is expressed relative to the standard obtained from cells cultivated in 2xYT medium.

B. Saturation ligand-binding assay was performed with membranes obtained from cells grown in (i) 2xYT medium, collected 40 hours post-induction; (ii) MSM supplemented with glucose and ammonium chloride, cells collected 10 hours post-induction; (iii) MSM supplemented with glucose, ammonium chloride and L-tryptophan, cells collected 4 hours post-induction.

Fig. 7.

A. Growth of *E. coli* BL21-130 cells and glucose consumption during the fed-batch fermentation. Ammonium chloride was added to the medium simultaneously with the addition of glucose, at a ratio 1: 4 (w/w). The fermentation temperature was kept at 37°C for 7 hours and was then decreased within 30 minutes to 20°C. At this point ¹⁵N₂ L-tryptophan was added. The induction with 1 mM IPTG was started 2.5 hours later. The cells were harvested 4 hours after induction.

B. Activity of CB2 protein expressed in MSM supplemented with glucose and ¹⁵N₂ labeled L-tryptophan. The levels of active receptor are compared to the positive control obtained from BL21-130 cells cultivated in 2x YT medium.

Fig. 8. (A) Instant-blue stained gel and (B) Western blot of the purified ^{15}N -Trp-labeled CB2 protein. (C) G protein activation on CB2 protein reconstituted into liposomes
CB2 was isolated from the biomass, purified and reconstituted into POPC-POPS liposomes as described in Materials and Methods.

A, Purified CB2 protein was mixed with loading buffer, resolved in SDS-PAGE and stained with Instant Blue (Expedion, Cambridge, UK).

B, 2 µg of purified CB2 protein was resolved in SDS-PAGE, blotted onto Nitrocellulose membrane, and probed with antibodies raised against N-terminal peptide of CB2 (Cayman Chemical, Ann Arbor, MI).

C, G protein activation assay was performed as described in Materials and Methods. Activity is presented as CPM of accumulated ^{35}S - γ -GTP per ng of CB2 in the reaction. As a positive control, *E. coli* membranes expressing CB2-130 protein are used. As yet another control, we have used an unlabeled CB2-130 protein reconstituted into POPC-POPS liposomes using the same procedure.

↓
SGSWSHPQFEKSGGASWSHPQFEKGGSEECWTEIA
NGSKDGLDSNPMKDYMLSGPQKTAVAVLCTLLGLLSALE
NVAVLYLILSSHQLRRKPSYLFIGSLAGADFLASVVFACSF
VNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDRY
LCLRYPPSYKALLTRGRALVTLGIMWVLSALVSYLPLMGW
TCCPRPCSELFPLIPNDYLLSWLLFIAFLFSGIITYGHVLW
KAHQHVASLSGHQDRQVPGMARMRLDVRLAKTLGLVLAV
LLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSM
VNPVIYALRSGEIRSSAHHCLAHWKKCVRGLGSEAKEEAP
RSSVTETEADGKITPWPDSRDLDLSDCGTHHHHHHHHHH
 ↑

Fig. 9.

Amino acid sequence of CB-130 protein after cleavage with TEV protease. N- and C-terminal amino acid residues of the native CB2 protein are marked with arrows. Transmembrane domains are highlighted in bold and peptides observed by LC-MS/MS analysis of CNBr + Trypsin digestion of CB2-130 protein are underlined. 70% sequence coverage was observed by LC-MS/MS.

Fig. 10.

High resolution mass spectrum of [¹⁵N] CB2-130 tryptic peptides; (A) SGSWSHPQFEK (+2) and (B) ITPWPDSR (+2). Experimental mass spectra are shown in top panels and the theoretical isotopic pattern for the light and heavy [¹⁵N] tryptophan containing peptides are shown in lower panels.

Fig. 11. ^{15}N -solid state NMR spectra of ^{15}N -Trp-CB2 (bottom trace) and of uniformly labeled ^{15}N , ^{13}C -CB2 (top trace). The ^{15}N -Trp-CB2 spectrum shows a single resonance with the chemical shift of ϵ - ^{15}N -Trp (the nitrogen atom of the indole ring).

Table 1

Concentration of amino acid groups in the different media

Medium	Group 1 (mg/l)	Group 2 (mg/l)	Group 3 (mg/l)	Addition of L-Trp at induction
AAM1	25	50	100	–
AAM2	100	200	400	–
AAM3	100	200	400	+

Table 2Effects of different media on cell growth (1st adaptation phase)

Medium	Citric acid concentration [g/L]	Specific growth rate [h ⁻¹]	Doubling time [h]	Final optical density
YT (2 x) *	0	1.13	0.6	4.48
M9 *	0	0.46	1.5	0.34
MSM *	0	0.39	1.8	0.74
MSM *	0.5	0.38	1.9	0.72
MSM *	1.0	0.39	1.8	0.69
MSM *	2.0	0.35	2.0	0.64
AAM 1	0	0.75	0.9	1.1
AAM 1 **	0	0.94	0.7	3.5

* supplemented with 1 g/L glucose

** supplemented with 2 g/L glucose

Table 3

Increase in BL21 (DE3) growth rates during adaptation to MSM

Adaptation phase	Specific growth rate [h^{-1}]	Doubling time [h]
1 st	0.39	1.8
2 nd	0.44	1.6
3 rd	0.53	1.3