



HAL
open science

Mammalian G protein-coupled receptor expression in *Escherichia coli*: II. Refolding and biophysical characterization of mouse cannabinoid receptor 1 and human parathyroid hormone receptor 1.

Kerstin Michalke, Céline Huyghe, Julie Lichière, Marie-Eve Gravière, Marina Siponen, Giuliano Sciara, Isabelle Lepaul, Renaud Wagner, Christine Magg, Rainer Rudolph, et al.

► To cite this version:

Kerstin Michalke, Céline Huyghe, Julie Lichière, Marie-Eve Gravière, Marina Siponen, et al.. Mammalian G protein-coupled receptor expression in *Escherichia coli*: II. Refolding and biophysical characterization of mouse cannabinoid receptor 1 and human parathyroid hormone receptor 1.. *Analytical Biochemistry*, 2010, 401 (1), pp.74-80. 10.1016/j.ab.2010.02.017. hal-01595269

HAL Id: hal-01595269

<https://hal.science/hal-01595269v1>

Submitted on 21 Feb 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Mammalian GPCR expression in *Escherichia coli*. Part 2: Refolding and Biophysical Characterization of muCB1R and huPTH1R

Kerstin Michalke^{1,4}, Céline Huyghe^{1,4}, Julie Lichière¹, Marie-Eve Gravière^{1,4}, Marina Siponen^{1,4}, Giuliano Sciara¹, Isabelle Lepaul², Renaud Wagner², Christine Magg³, Rainer Rudolph³, Christian Cambillau^{*1}, Aline Desmyter^{*1}.

1. Architecture et Fonction des Macromolécules Biologiques, UMR 6098, CNRS and Universités of Marseille, Case 932, 163 Ave de Luminy, F-13288 Marseille Cedex 09, France.

2. IREBS, FRE3211-CNRS/UdS Institut de Recherche de l'Ecole Supérieure de Biotechnologie de Strasbourg, Blv Sébastien Brant, BP 10413, F-67412 Illkirch Cedex, France.

3. Department of Biotechnology, Martin-Luther University, Kurt-Mothes street 3, D-06120 Halle/Saale, Germany.

4. BioXtal, 2, rue Thomas Edison, 67450 Mundolsheim, France.

*Corresponding authors

Tel: +33.491.82.55.91/90; Fax: +33.491.16.45.36

e-mail: desmyter@afmb.univ-mrs.fr or cambillau@afmb.univ-mrs.fr

We dedicate this work to the memory of Prof. Rainer Rudolph, who died in November 2009.

Running Title: Purification and characterization of two GPCRs refolded from inclusion bodies.

Abbreviations: GPCR; G protein-coupled receptor, muCB1R; mouse cannabinoid receptor, huPTH1R; human parathyroid hormone receptor, TEV; Tobacco etch virus, IPTG; isopropyl- β -D-thiogalactoside, HRP; horse radish peroxidase, PMSF; phenylmethylsulfonyl fluoride, SDS; sodium dodecyl sulfate, DTT; dithiothreitol, EDTA; ethylenediaminetetraacetic acid, GSH and GSSH; L-reduced and oxidized glutathione, DDM; n-dodecyl- β -D-maltopyranoside, Cymal 6; 6-Cyclohexylhexyl- β -D-maltoside, SPR; Surface Plasmon Resonance, K_d ; equilibrium dissociation constant.

Abstract

G protein-coupled receptors (GPCRs) represent ~3% of the human proteome. They are involved in a large number of diverse processes and are therefore the most prominent class of pharmacological targets. Besides rhodopsin, X-ray structures of classical GPCRs have only recently been resolved, including the β 1 and β 2 adrenergic receptors and the A2A adenosine receptor. This lag in obtaining GPCR structures is due to several tedious steps that are required before beginning the first crystallization experiments: protein expression, detergent solubilization, purification and stabilization. With the aim to obtain active membrane receptors for functional and crystallization studies, we first have recently reported a screen of expression conditions for ~100 GPCRs in *E. coli*, providing large amounts of inclusion bodies, a prerequisite for the subsequent refolding step (Michalke *et al.* 2009 Anal. Biochem. 386,147-155). Here we report a novel artificial chaperone-assisted refolding procedure adapted for the GPCR inclusion body refolding, followed by protein purification and characterization. The refolding of two selected targets, the mouse cannabinoid receptor 1 (muCB1R) and the human parathyroid hormone receptor 1 (huPTH1R), was achieved from solubilized receptors using detergent and cyclodextrin as protein folding assistants. We could demonstrate excellent affinity of both refolded and purified receptors for their respective ligands. In conclusion, this study suggests that the procedure described here can be widely used to refold GPCRs expressed as inclusion bodies in *E. coli*.

Keywords: G protein-coupled receptor, refolding, protein expression.

Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins characterized by a conserved fold of seven transmembrane helices connected by highly variable extracellular loops, an extracellular N-terminus and a cytoplasmic C-terminus. They are distributed into three main subfamilies according to sequence homology, receptor function and ligand nature [1]. GPCRs are involved in the majority of cellular responses to hormones and neurotransmitters as well as in the processes of sight, smell and taste. They represent the largest, most versatile group of membrane receptors and also the most pharmaceutically important, accounting for about 30% of all human drug targets for a wide range of diseases, including cancer and cardiovascular, metabolic, central nervous system and inflammatory diseases [2]. However, because there are still experimental difficulties associated with their production, purification and crystallization, only a limited number of GPCR structures have been solved so far [3; 4; 5; 6; 7].

In this study, we focused on two GPCRs, the mouse cannabinoid receptor (muCB1R) and the human parathyroid hormone receptor (huPTH1R), as representatives of the class A (or rhodopsin-like) and B (or secretin-like) families, respectively. The cannabinoid type 1 receptor is mostly expressed in the central nervous system at nerve terminals and on axons, where it affects neural excitability and modulates neurotransmission [8]. Cannabinoid ligands, which are small hydrophobic molecules, bind within the hydrophobic core formed by the transmembrane helices. An aromatic cluster formed by residues of helices 3, 4 and 5 was proposed to constitute the binding site for cannabinoid receptor agonists and antagonists [9]. The N-tail of the receptor, although unusually long (116 aa) compared to the class A family, does not seem to be important either for ligand binding or for receptor activation [10]. The parathyroid hormone type 1 receptor, which is highly expressed in kidney and bone cells, plays a central role in the regulation of blood phosphate and calcium concentrations [11]. The binding site for human parathyroid hormone (84 aa) or its N-terminal fragment (34 aa) [12] includes the N-terminus (170 aa) and extracellular loops that join the transmembrane domains of the receptor. A two-site binding model for the PTH(1-34) peptide proposed that the N-terminal part (1-15) interacts with the membrane-embedded portions of the receptor and the intervening loops, resulting in PTH1R activation, whereas the C-terminal part (16-34) is required for high affinity binding to the N-terminal domain of the receptor [13]. Proteins overexpressed in *E. coli* are often obtained as insoluble inclusion bodies, and GPCR expression in that system is no exception [14]. In this study, both receptors, muCB1R and huPTH1R, were produced at high quantities as insoluble inclusion bodies in *E. coli*. As a consequence, a refolding procedure was necessary in order to obtain the native proteins.

Although the refolding of globular proteins from inclusion bodies has now become routine [15; 16], only a few examples of the *in vitro* renaturation of membrane proteins have been reported [17; 18; 19; 20]. On the other hand, the prokaryotic GroEL chaperone has been shown to have a role in protein folding and removal of aggregates [21]. Artificial chaperone-assisted refolding, a two-step process inspired by the mechanism of the GroEL chaperone system, has been reported to efficiently refold denatured proteins [22; 23; 24]. After producing large quantities of protein in inclusion bodies, we report here the refolding of the inclusion bodies of two GPCRs using an optimized artificial chaperone-assisted refolding procedure.

Material and methods

The *E. coli* strain Rosetta(DE3)pLysS and pET15b plasmid were obtained from Novagen, (Madison, WI), and the C43(DE3) strain was from Avidis SA [25]. The pET15N2 vector is a derivative of pET15b in which the *NcoI/BamHI* fragment was replaced by a 10-Histidine tag and a TEV protease cleavage sequence upstream of the *BamHI/SpeI* cloning site [26]. Ni-NTA superflow resin, 6xHis ladder and penta-His-HRP conjugate were from Qiagen. The ECL chemiluminescent detection system, chromatography columns and equipment were purchased from Amersham Biosciences. PVDF membrane was from Roche. Detergents were from Anatrace, methyl- β -cyclodextrin was from Fluka, GSH and GSSG were from Sigma, and the bicinchoninic acid (BCA) protein assay kit was from Pierce. Amicon-Ultra cutoff 10-30 kDa concentrators were from Millipore, and dialysis membranes (ZelluTrans) were from Roth. CP55940 was purchased from Tocris Bioscience, [3 H]-CP55940 from Perkin Elmer and [3 H]-SR141716A from Amersham Biosciences. The PTH(1-34) peptide (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF) was synthesized by GeneCust. Whatman GF/B glass fiber filters were from Whatman.

Shaking flasks and fermentor E. coli GPCR expression:

C43(DE3) and Rosetta(DE3)pLysS *E. coli* strains transformed with the pET15N2/muCB1R and pET15b/huPTH1R vectors, respectively, were grown in Luria-Bertani (LB) medium supplemented with 100 μ g/mL ampicillin (and 34 μ g/mL chloramphenicol for Rosetta(DE3)pLysS) at 37°C overnight. Precultures were transferred to 1 L LB medium in shaking flasks and grown until an OD₆₀₀ value of 0.7-0.8 was reached. The expression of recombinant GPCRs was induced by the addition of 1 mM IPTG at 37°C for 3 H. For fermentation, 8 L of yeast extract medium (50 g/L yeast extract, 0.5 g/L ammonium chloride, 5 g/L glucose, 11 g/L di-potassium hydrogen phosphate and 0.68 g/L magnesium sulfate)

supplemented with 100 µg/mL ampicillin in a 12 L working volume fermentor was inoculated with a preculture grown at 37°C. Cells were grown at 37°C, at constant pH of 7.0, to an OD₆₀₀ between 7 and 15 with constant stirring (800-1000 rpm) and an air supply of 1-2 volumes per minute before applying feedback with a solution of 30% yeast extract and 25% glycerol. The culture was induced with 1 mM IPTG when an OD₆₀₀ of 40 was reached and incubated for 3 to 4 h (OD₆₀₀ ~60-80). Cells were harvested at 4°C by centrifugation, and pellets were either stored at -20°C or resuspended in lysis buffer.

Inclusion body solubilization and Ni-NTA purification:

Five grams of wet pellet obtained from ~1.5 L of bacterial culture in shaking flasks or from ~0.1 L in fermentors was resuspended in 100 ml of lysis buffer (0.1 M Tris-HCl pH 7.5, 10 mM EDTA, 1 mM PMSF, 0.25 mg/mL lysozyme, 10 µg/mL DNaseI and 20 mM MgCl₂) and incubated for 1 h at room temperature prior to French press disruption at 830 bar (French® press, Thermo Scientific). The sample was centrifuged at 48000g for 20 min at 4°C; the pellet was resuspended in 0.1 M Tris-HCl pH 7.5, 10 mM EDTA and 1 M NaCl and homogenized (Homogeniser DI 18 Yellow Line; Dutscher). Inclusion bodies were recovered at 4°C by centrifugation at 48000g for 20 min and washed twice with 0.1 M Tris-HCl pH 7.5 and 1 mM EDTA. Washed inclusion bodies were finally solubilized in 40 mL 0.1 M Tris-HCl pH 8.0, 20 mM SDS, 100 mM DTT and 1 mM EDTA overnight at room temperature. Solubilized proteins were clarified from the insoluble material by centrifugation at 48000g for 20 min at room temperature and dialyzed against 0.1 M sodium phosphate pH 8.0 and 10 mM SDS. Solubilized inclusion bodies were applied to a 10 ml Ni²⁺ affinity column (Ni-NTA Superflow, Qiagen) equilibrated in 0.1 M sodium phosphate pH 8. After five successive column volume washes in 0.1 M sodium phosphate and 10 mM SDS at pH 8.0 and pH 7.0, the receptor was eluted in the same buffer at pH 6.0. Fractions of the eluted peak were pooled and analyzed by SDS-PAGE and Western blot, and the protein concentration was determined by UV spectrometry from absorbance at 280 nm.

GPCR refolding and GF purification:

Ni fractions were pooled and dialyzed against 0.1 M Tris-HCl pH 8.0, 0.5 mM EDTA and 10 mM SDS for muCB1R or 0.1 M Tris-acetate pH 4.5, 0.5 mM EDTA and 10 mM SDS for huPTH1R. Both samples were diluted to 0.5 mg/ml in the final buffer 0.1 M Tris-HCl pH 8.0, 3-5 mM SDS and 0.5 mM EDTA before the addition with stirring of 5 mM L-reduced glutathione (GSH), 1 mM oxidized glutathione (GSSG), 6-10 mM n-dodecyl-β-D-maltopyranoside (DDM), 6-10 mM 6-Cyclohexylhexyl-β-D-maltoside (Cymal 6) and 6-8 mM

methyl- β -cyclodextrin. After 36-60 h at -20°C , the sample was allowed to defrost at 4°C before addition of 25 mM CaCl_2 and incubation overnight at 4°C with stirring. Aggregates were removed by centrifugation at 20000g for 15 min at 4°C . Refolded muCB1R was first concentrated to 1.5-2 mg/ml on a Amicon-Ultra 10 kDa cutoff concentrator prior to loading onto a HiLoad 16/60 or 10/30 Superdex75 gel filtration column in 10 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DDM and 1 mM Cymal 6. Refolded huPTH1R was loaded at 0.5 mg/ml onto a HiLoad 16/60 Superdex75 gel filtration column in 100 mM Tris-HCl pH 7.6, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DDM and 1 mM Cymal 6. Protein concentrations were determined by UV spectrometry from absorbance at 280 nm or by the BCA method using bovine serum albumin as a standard. The SDS concentration was determined by SDS/methylene blue ion pair extraction into chloroform [27]. Purified proteins were always kept at 4°C before characterization steps.

Receptor characterization:

Western blot: Proteins were transferred from a 15% SDS-PAGE gel to Hybond PVDF membrane, which was blocked with 5% non-fat dried milk in 20 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.1% Tween 20 at 4°C . The filter was then treated with anti-His-HRP primary antibodies. GPCR-specific bands were visualized by ECL chemiluminescence.

Mass spectrometry: Mass spectrometry was performed on a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Bruker Autoflex, Bruker Daltonics, Bremen, Germany) according to standard procedures [28]. Peptide mass finger printing was done by in-gel trypsin digestion and subsequent analysis of the peptides by MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using standard protocols [29].

MALS/UV/RI (Wyatt): Size exclusion chromatography was performed on an Alliance 2695 HPLC system (Waters) using a Silica Gel KW804 column (Shodex) equilibrated with 100 mM Tris-HCl pH 7.0, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DDM and 1 mM Cymal 6 at a flow rate of 0.5 ml/min. Thirty microliters of sample at approximately 0.5 mg/ml in the appropriate buffer containing detergents was applied to the column. Detection was performed using a triple-angle light scattering detector (Mini-DAWNTM TREOS, Wyatt technology, Santa Barbara, USA). Molecular weight determination was performed by the ASTRA V software (Wyatt technology).

Radioligand binding assays:

The purified refolded muCB1R (10 pg) was incubated with varying concentrations of the radioligands [^3H]-CP55940 (0.46-37 nM) and [^3H]-SR141716A (0.5-32 nM) in binding buffer

(10 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DDM, 1 mM Cymal 6 and 10 mM MgCl₂) until equilibrium was reached. Non-specific binding was determined in the presence of an excess of unlabeled ligand (100 μM CP55940). All assays were performed in triplicate in a total volume of 200 μl per tube. After incubation for 2 h at room temperature, bound and free ligands were separated by rapid vacuum filtration through 0.3% polyethyleneimine presoaked Whatman GF/B glass fiber filters. After the filters were washed three times in cold 50 mM Tris-HCl pH 7.4, the retained radioactivity was measured by liquid scintillation counting (Tri-Carb, Packard). Saturation curves were analyzed by non-linear regression using PRISM (Synergy Software) for determination of the B_{max} and K_d values.

Fluorometry binding assays:

Tryptophan fluorescence quenching experiments were done on a Varian Eclipse spectrofluorometer (Cary Eclipse) using a quartz cuvette in a right-angle configuration (light paths were 0.4 cm and 1 cm for excitation and emission, respectively). The excitation wavelength was 290 nm, and emission spectra were recorded in the range of 300 to 420 nm. Titrations were carried out at room temperature using 100 nM of purified refolded huPTH1R in 100 mM Tris-HCl pH 7.6, 300 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DDM and 1 mM Cymal 6 in the presence of 10 to 500 nM of the ligand PTH(1-34). The buffer and PTH(1-34) contributions were subtracted under the same experimental conditions. Saturation curves were generated using fluorescence values at 340 nm and analyzed with PRISM (Synergy Software).

SPR affinity measurements:

Kinetic analyses of the GPCR/ligand interactions were performed on a BIAcore 1000 instrument (BIAcore, Uppsala, Sweden) using a CM5 (carboxymethylated dextran) sensor chip coated with 500-900 resonance units of the ligand PTH(1-34) immobilized by amine coupling. Non-specific ligand for huPTH1R (an antibody fragment of 15 kDa specific of muCB1R) was coated on a control flow-cell to subtract the nonspecific binding signal. For the determination of kinetic constants, dilutions of huPTH1R (75-600 nM in 100 mM Tris-HCl pH 7.6, 300 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DDM and 1 mM Cymal 6) were passed over the ligand-coated flow-cell and the control flow-cell. Binding traces were recorded in triplicate for at least five concentrations of analyte. Each binding-regeneration cycle was performed at room temperature with a constant flow rate of 40 μL/min. In each cycle, buffer (50 μL) was injected first to stabilize the baseline, the analyte (80 μL) was then injected, and the spontaneous dissociation was followed for 6 minutes. Binding surfaces were

regenerated by injection of 5 μ L of 0.5% SDS and further washing with running buffer for 6 minutes. Data were evaluated with the BIAevaluation software (BIAcore, Uppsala, Sweden) according to the (1:1) Langmuir binding model.

Results and discussion

***E. coli* expression, inclusion body solubilization and purification of huPTH1R and muCB1R:**

We previously have shown the production of a high quantity of GPCRs as insoluble inclusion bodies in *E. coli* [14]. In this study, the sub-cloned mouse cannabinoid receptor (muCB1R) and human parathyroid hormone receptor (huPTH1R) were screened for expression in *E. coli* by varying the vectors, strains and induction temperatures (data not shown). Both receptors were then produced as recombinant proteins following the optimized expression conditions, *i.e.*, in T7 promoter-based vectors containing either a N-terminal deca- or hexa-histidine tag and in either C43(DE3) or Rosetta(DE3)pLysS *E. coli* strains at 37°C (see Material and Methods). Analysis of the bacterial lysate showed that in these conditions, the two receptors accumulated exclusively in the insoluble fraction as misfolded proteins in inclusion bodies with no indication of any membrane association. Reduced toxicity associated with expression in inclusion bodies allowed high fermentation rates to be achieved with the recovery of ~100 g cell paste/L of culture. Receptors were recovered from inclusion bodies under denaturing conditions using the anionic sodium dodecyl sulfate (SDS) detergent as the solubilizing agent and later purified by immobilized metal affinity chromatography on a Ni²⁺-NTA column. Almost pure proteins at yields between 400 and 600 mg/L of fermentor culture were routinely obtained because one major band of the expected sizes corresponding to 55.6 kDa for muCB1R and 66.5 kDa for huPTH1R was on SDS-PAGE (Fig. 1A and 1B, a). The identities of the receptors were confirmed by anti-His Western blot analysis (Fig. 1A and 1B, b) and peptide mass fingerprinting. MALDI/TOF analysis of the peptides resulting from trypsin digestion of the SDS-PAGE bands revealed 13 and 9 peptides from muCB1R and huPTH1R, corresponding to 33% and 22% sequence coverage, respectively (data not shown).

huPTH1R and muCB1R refolding and purification:

In order to obtain the native proteins, an artificial chaperone-assisted refolding procedure was developed based on the idea that a detergent plays the role of a chaperone and assures that the protein does not aggregate before it reaches its native form [22; 23; 24]. Here, renaturation of

the solubilized receptors was performed by exchange of SDS (the artificial chaperone) to a mixture of the non-ionic detergents n-dodecyl- β -D-maltoside (DDM) and cyclohexyl-n-hexyl- β -D-maltoside (Cymal 6) (see Methods). The refolding process was done in the presence of methyl- β -cyclodextrin, a cyclic oligosaccharide with seven glucose units. This amphiphilic compound possesses a hydrophobic cavity in which hydrophobic molecules of the appropriate size may bind; this property was used here to strip-off the SDS [30]. By contacting the bulky hydrophobic amino acid chains, cyclodextrin itself may also possess some artificial chaperone properties by increasing the stability of folding intermediates [31]. Finally, to ensure that the correct disulfide bridges were regenerated, which are crucial for ligand binding to both receptors [32], reduced and oxidized glutathione (GSH, GSSG) were also added to the refolding buffer as “oxido-shuffling” reagents [16]. After renaturation, a final purification step consisting in a size exclusion chromatography was performed to remove the excess of methyl- β -cyclodextrin/SDS. Refolded proteins were recovered almost pure, as determined by SDS-PAGE (Fig. 1A and 1B, c). Besides the major bands around 55 and 66 kDa corresponding to the respective monomeric receptors, several bands of higher or lower molecular weight were observed on the SDS-PAGE and anti-His Western blots, which may indicate a small amount of contaminating material, oligomeric forms of the receptors (see below) or truncated receptors (Fig. 1A and 1B, c,d). An average of 300-350 mg protein/L of culture was recovered for the refolded and purified receptors, which corresponds to at least 50% of the initial solubilized metal affinity purified inclusion body protein material. The sample SDS concentration at the final step was systematically measured (see Methods) and was always below 25 μ M. The addition of 10% glycerol, 300 mM NaCl and 0.05% DDM and Cymal 6 in the gel filtration buffer helped to stabilize the receptor and enabled us to purify enough SDS-free receptor material for further characterization procedures.

huPTH1R and muCB1R characterization:

Freshly refolded and purified muCB1R and huPTH1R were subjected to analytical size exclusion chromatography on an HPLC-Wyatt system. Chromatograms revealed two minor peaks, around 19 minutes for muCB1R (Fig. 2C) and 17 minutes for huPTH1R (Fig. 2A). Although the corresponding fractions showed polydispersity in the DDM and Cymal 6 detergent mixture, they contained molecules of an approximate total mass of respectively 176 and 190 kDa, which corresponds to the dimeric forms of the receptors. A major peak at 12 minutes could also be observed in both chromatograms, corresponding to aggregates or higher order oligomers with approximate calculated masses above 10^3 kDa (Fig. 2A and C – light lines). In an attempt to stabilize both receptors, their respective ligands, CP55940 and PTH(1-

34), were added during the refolding step, but this did not show an effect on aggregation (data not shown). As well, no effect on aggregation could be shown varying slightly the ratio of detergents and methyl- β -cyclodextrin or omitting the calcium addition in the refolding procedure (data not shown). On the other hand, an additional ultracentrifugation step allowed us to almost completely eliminate aggregates in the samples (Fig. 2A and C – dark lines). By means of light scattering, refraction and UV analysis of the centrifuged samples, the molecular weight distribution inside the respective receptor peaks could be determined and confirmed the presence of a mixture of receptor oligomers between 100 and 1000 kDa (Fig. 2B and D). Among these populations, a portion of the mouse cannabinoid CB1R appears as a dimer of 110 kDa with a contribution of 66 kDa for detergents and the human parathyroid PTH1R as a dimer of 130 kDa with a contribution of 60 kDa for detergents.

Binding studies with muCB1R:

The functional activity of the refolded and purified muCB1R was assessed by measuring direct binding to its radiolabeled ligand. muCB1R gave a maximum binding capacity (B_{\max}) of 5433 ± 1343 pmol mg^{-1} for the agonist [^3H]-CP55940 and 5309 ± 686 pmol mg^{-1} for the inverse agonist [^3H]-SR141716A, suggesting that about 30% of the refolded receptor was active. The calculated equilibrium dissociation constants (K_d) were 19.4 ± 9.7 nM and 38.2 ± 7.8 nM, respectively (Fig. 3). Although these values are 10 times higher than values described for membrane embedded CB1R [33], they are still satisfactory since decreased ligand affinity after GPCR solubilization and purification seems to be a rather widespread phenomenon [34; 35]. A possible explanation might be that lower lateral pressure on the transmembrane helices due to the lack of a native lipid bilayer around the receptor has an effect on the binding pocket. Since it is known that cannabinoid receptors bind their ligands inside the helix bundle [9], they might be especially susceptible to conformational changes in that region.

Binding studies with huPTH1R:

The interaction of huPTH1R with the agonist parathyroid hormone peptide (1-34) was first monitored by recording the quenching of the intrinsic receptor fluorescence upon addition of ligand. The calculated equilibrium dissociation constant (K_d) of 56 ± 5.9 nM (Fig. 4) was in the same range as values given for solubilized PTHR [35]. To evaluate the stability of the receptor, binding experiments were repeated 2 and 6 days following receptor purification. Tryptophan fluorescence dropped from 35% on day 1 to 25% and 7% on days 2 and 6, respectively. At the same time, the dissociation constant remained at 50 ± 7.8 nM after 2 days but dropped drastically to 523 ± 106.3 nM after 6 days (Fig. 4).

In parallel, the dissociation constant of refolded purified huPTH1R with respect to ligand binding was determined by Surface Plasmon Resonance. The PTH(1-34) peptide of 4.1 kDa was covalently coupled to a CM5 chip by amine coupling. Immobilization of the ligand was preferred to the receptor because it is more stable, allowing for a rapid and easy regeneration procedure using SDS. Moreover, the larger molecular weight of the GPCR makes it a more favorable candidate for the analyte. An example of the binding curves for the receptor to the ligand is depicted in Figure 5. The equilibrium dissociation constant of the ligand for huPTH1R was found to be in the nanomolar range (8.4 nM on average). Depending on the time at which the binding was measured (4 to 48 h after receptor purification), values varying between 0.5 and 16 nM were observed. This confirmed the instability of huPTH1R over time, which was already observed in the fluorescence measurements (see above). Nevertheless, these values were better than those described for solubilized PTHR and similar to the binding values of the ligand to membrane-embedded huPTH1R (between 2 and 16 nM depending on the mammalian cells used) [35; 36; 37].

Conclusions

Following the first step of large-scale inclusion body production, we performed refolding studies and characterization. To this end, we have used and optimized a procedure to refold *E. coli* overexpressed GPCRs using SDS and β -cyclodextrin as protein folding assistants. In the first step, SDS was used to strongly inhibit aggregation and solubilize the inclusion bodies before Ni^{2+} affinity purification. In the second step, β -cyclodextrin, an efficient SDS stripping agent, was added to allow the solubilized protein to regain its native conformation. These experiments were done in the presence of non-ionic detergents, which helped to maintain a functional folded state of the membrane proteins outside their native membrane environment. As a result, we obtained good quantities of refolded active GPCRs, namely, human PTH1R and mouse CB1R. These experiments make it possible to perform crystallization experiments with these two important targets. They also offer a faster and cheaper alternative to GPCR production in eukaryotic systems.

Acknowledgements: We thank the structural genomics team of the AFMB (Architecture et Fonction des Macromolécules Biologiques) laboratory for technical assistance. This study was supported by the European Union Program EMEP (Cooperative program for Monitoring and Evaluation of the Long-Range Transmission of Air Pollutants in Europe, FP6 Contract LSHG-CT2004-504601), by the MePNet (Membrane Protein Network) program of BioXtal,

by the Marseille-Nice Genopole and by the CNRS Program "Protéomique et Génie des Protéines". Dr. Kenneth Lündström and Etienne L'Hermite are acknowledged for support and interest. We thank J. Oschmann for the set-up of first refolding experiments on huPTH1R, V. Campanacci for her help in fluorometry experiments and M. Tegoni for her help with the SPR experiment analysis.

References

- [1]J. Bockaert, and J.P. Pin, Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* 18 (1999) 1723-9.
- [2]G.C. Terstappen, and A. Reggiani, In silico research in drug discovery. *Trends Pharmacol Sci* 22 (2001) 23-6.
- [3]V.P. Jaakola, M.T. Griffith, M.A. Hanson, V. Cherezov, E.Y. Chien, J.R. Lane, A.P. Ijzerman, and R.C. Stevens, The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322 (2008) 1211-7.
- [4]T. Warne, M.J. Serrano-Vega, J.G. Baker, R. Moukhametzianov, P.C. Edwards, R. Henderson, A.G. Leslie, C.G. Tate, and G.F. Schertler, Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454 (2008) 486-91.
- [5]S.G. Rasmussen, H.J. Choi, D.M. Rosenbaum, T.S. Kobilka, F.S. Thian, P.C. Edwards, M. Burghammer, V.R. Ratnala, R. Sanishvili, R.F. Fischetti, G.F. Schertler, W.I. Weis, and B.K. Kobilka, Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450 (2007) 383-7.
- [6]V. Cherezov, D.M. Rosenbaum, M.A. Hanson, S.G. Rasmussen, F.S. Thian, T.S. Kobilka, H.J. Choi, P. Kuhn, W.I. Weis, B.K. Kobilka, and R.C. Stevens, High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318 (2007) 1258-65.
- [7]K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, and M. Miyano, Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289 (2000) 739-45.
- [8]T.F. Freund, I. Katona, and D. Piomelli, Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* 83 (2003) 1017-66.
- [9]S.D. McAllister, Q. Tao, J. Barnett-Norris, K. Buehner, D.P. Hurst, F. Guarnieri, P.H. Reggio, K.W. Nowell Harmon, G.A. Cabral, and M.E. Abood, A critical role for a tyrosine residue in the cannabinoid receptors for ligand recognition. *Biochem Pharmacol* 63 (2002) 2121-36.
- [10]H. Andersson, A.M. D'Antona, D.A. Kendall, G. Von Heijne, and C.N. Chin, Membrane assembly of the cannabinoid receptor 1: impact of a long N-terminal tail. *Mol Pharmacol* 64 (2003) 570-7.
- [11]H. Juppner, A.B. Abou-Samra, M. Freeman, X.F. Kong, E. Schipani, J. Richards, L.F. Kolakowski, Jr., J. Hock, J.T. Potts, Jr., H.M. Kronenberg, and et al., A G protein-linked

receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* 254 (1991) 1024-6.

[12]M. Shimizu, J.T. Potts, Jr., and T.J. Gardella, Minimization of parathyroid hormone. Novel amino-terminal parathyroid hormone fragments with enhanced potency in activating the type-1 parathyroid hormone receptor. *J Biol Chem* 275 (2000) 21836-43.

[13]T.J. Gardella, and H. Juppner, Molecular properties of the PTH/PTHrP receptor. *Trends Endocrinol Metab* 12 (2001) 210-7.

[14]K. Michalke, M.E. Graviere, C. Huyghe, R. Vincentelli, R. Wagner, F. Pattus, K. Schroeder, J. Oschmann, R. Rudolph, C. Cambillau, and A. Desmyter, Mammalian G-protein-coupled receptor expression in *Escherichia coli*: I. High-throughput large-scale production as inclusion bodies. *Anal Biochem* 386 (2009) 147-55.

[15]R. Vincentelli, S. Canaan, V. Campanacci, C. Valencia, D. Maurin, F. Frassinetti, L. Scappucini-Calvo, Y. Bourne, C. Cambillau, and C. Bignon, High-throughput automated refolding screening of inclusion bodies. *Protein Sci* 13 (2004) 2782-92.

[16]R. Rudolph, and H. Lilie, In vitro folding of inclusion body proteins. *FASEB J* 10 (1996) 49-56.

[17]H. Kiefer, J. Krieger, J.D. Olszewski, G. Von Heijne, G.D. Prestwich, and H. Breer, Expression of an olfactory receptor in *Escherichia coli*: purification, reconstitution, and ligand binding. *Biochemistry* 35 (1996) 16077-84.

[18]H. Rogl, K. Kosemund, W. Kuhlbrandt, and I. Collinson, Refolding of *Escherichia coli* produced membrane protein inclusion bodies immobilised by nickel chelating chromatography. *FEBS Lett* 432 (1998) 21-6.

[19]J.L. Baneres, D. Mesnier, A. Martin, L. Joubert, A. Dumuis, and J. Bockaert, Molecular characterization of a purified 5-HT₄ receptor: a structural basis for drug efficacy. *J Biol Chem* 280 (2005) 20253-60.

[20]A. Bazarsuren, U. Grauschopf, M. Wozny, D. Reusch, E. Hoffmann, W. Schaefer, S. Panzner, and R. Rudolph, In vitro folding, functional characterization, and disulfide pattern of the extracellular domain of human GLP-1 receptor. *Biophys Chem* 96 (2002) 305-18.

[21]J.P. Hendrick, and F.U. Hartl, Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem* 62 (1993) 349-84.

[22]Rozema, and Gellman, Artificial Chaperones: Protein refolding via sequential use of detergent and cyclodextrin. *J. Am. Chem. Soc.* 117 (1995) 2373-2374.

[23]D. Rozema, and S.H. Gellman, Artificial chaperone-assisted refolding of carbonic anhydrase B. *J Biol Chem* 271 (1996) 3478-87.

[24]D.L. Daugherty, D. Rozema, P.E. Hanson, and S.H. Gellman, Artificial chaperone-assisted refolding of citrate synthase. *J Biol Chem* 273 (1998) 33961-71.

- [25]B. Miroux, and J.E. Walker, Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260 (1996) 289-98.
- [26]K. Lundstrom, R. Wagner, C. Reinhart, A. Desmyter, N. Cherouati, T. Magnin, G. Zeder-Lutz, M. Courtot, C. Prual, N. Andre, G. Hassaine, H. Michel, C. Cambillau, and F. Pattus, Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. *J Struct Funct Genomics* 7 (2006) 77-91.
- [27]M. Arand, T. Friedberg, and F. Oesch, Colorimetric quantitation of trace amounts of sodium lauryl sulfate in the presence of nucleic acids and proteins. *Anal Biochem* 207 (1992) 73-5.
- [28]M. Cadene, and B.T. Chait, A robust, detergent-friendly method for mass spectrometric analysis of integral membrane proteins. *Anal Chem* 72 (2000) 5655-8.
- [29]A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 68 (1996) 850-8.
- [30]F. Couthon, E. Clottes, and C. Vial, Refolding of SDS- and thermally denatured MM-creatine kinase using cyclodextrins. *Biochem Biophys Res Commun* 227 (1996) 854-60.
- [31]Cooper, Effect of cyclodextrins on the thermal stability of globular proteins. *J. Am. Chem. Soc.* 114 (1992) 9208-9209.
- [32]U. Grauschopf, H. Lilie, K. Honold, M. Wozny, D. Reusch, A. Esswein, W. Schafer, K.P. Rucknagel, and R. Rudolph, The N-terminal fragment of human parathyroid hormone receptor 1 constitutes a hormone binding domain and reveals a distinct disulfide pattern. *Biochemistry* 39 (2000) 8878-87.
- [33]C.R. Chillakuri, C. Reinhart, and H. Michel, C-terminal truncated cannabinoid receptor 1 coexpressed with G protein trimer in Sf9 cells exists in a precoupled state and shows constitutive activity. *FEBS J* 274 (2007) 6106-15.
- [34]B.K. Lee, K.S. Jung, C. Son, H. Kim, N.C. VerBerkmoes, B. Arshava, F. Naidler, and J.M. Becker, Affinity purification and characterization of a G-protein coupled receptor, *Saccharomyces cerevisiae* Ste2p. *Protein Expr Purif* 56 (2007) 62-71.
- [35]L. Gan, J.M. Alexander, A. Wittelsberger, B. Thomas, and M. Rosenblatt, Large-scale purification and characterization of human parathyroid hormone-1 receptor stably expressed in HEK293S GnTI- cells. *Protein Expr Purif* 47 (2006) 296-302.
- [36]M. Shimada, X. Chen, T. Cvrk, H. Hilfiker, M. Parfenova, and G.V. Segre, Purification and characterization of a receptor for human parathyroid hormone and parathyroid hormone-related peptide. *J Biol Chem* 277 (2002) 31774-80.
- [37]M. Pines, A.E. Adams, S. Stueckle, R. Bessalle, V. Rashti-Behar, M. Chorev, M. Rosenblatt, and L.J. Suva, Generation and characterization of human kidney cell lines stably

expressing recombinant human PTH/PTHrP receptor: lack of interaction with a C-terminal human PTH peptide. *Endocrinology* 135 (1994) 1713-6.

Legends to figures

Fig. 1: SDS-PAGE (a and c) and anti-His Western blots (b and d) of muCB1R and huPTH1R, respectively. Molecular weight marker (M), total cell lysate (T), SDS solubilized inclusion bodies (S), flow through after Ni²⁺ column (FT), eluted solubilized receptor after Ni²⁺ column (Ni), purified refolded receptor after gel filtration (GF). Bands corresponding to the monomeric muCB1R (55.6 kDa) and huPTH1R (66.5 kDa) are marked with red arrows.

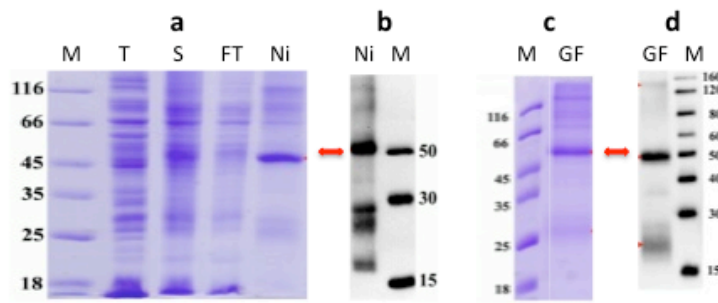
Fig. 2: HPLC-Wyatt analysis of purified huPTH1R (A and B) and muCB1R (C and D). **A and C:** Chromatograms of huPTH1R and muCB1R, respectively, before (light grey) and after (dark grey) ultracentrifugation. **B and D:** Enlargement of the absorption peaks corresponding to the respective receptors after ultracentrifugation. Contributions of the detergent micelle (light blue) and the receptor (red) to the total mass (blue) are depicted. The X-axis indicates the time scale of the HPLC injection, and the Y-axis indicates the UV absorbance at 280 nm (A and C) or the molar mass in kDa (B and D)

Fig. 3: Radioligand binding assays of refolded purified muCB1R expressed in E. coli. Various concentrations of **(A)** the agonist [³H]-CP55940 and **(B)** the inverse agonist [³H]-SR141716A (X-axis) were incubated with a fixed concentration of receptor protein (see Methods). The specific binding is presented as picomoles of active receptor per mg of total receptor subjected to the binding assay. The results shown are from three independent experiments.

Fig. 4: Saturation isotherms of PTH(1-34) ligand binding to huPTH1R determined by tryptophan quenching. Binding was measured 1 day (squares), 2 days (triangles) and 6 days (circles) after receptor purification. The receptor was titrated with PTH(1-34) (X-axis). The Y-values were obtained using $y=1-y$ using normalized Y-values of fluorescence intensity with the PRISM software.

Fig. 5: Binding assays of the refolded huPTH1R by surface plasmon resonance. An example of overlaid sensorgrams depicting the binding of different concentrations (between 75 and 300 nM) of the receptor huPTH1R on a flow-cell coated with PTH(1-34) ligand.

A. muCB1R



B. huPTH1R

