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A reconstitution protocol for the *in vitro* folded human G protein-coupled Y_2 receptor into lipid environment

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ABSTRACT

Although highly resolved crystal structures of G protein-coupled receptors have become available within the last decade, the need for studying these molecules in their natural membrane environment, where the molecules are rather dynamic, has been widely appreciated. Solid-state NMR spectroscopy is an excellent method to study structure and dynamics of membrane proteins in their native lipid environment. We developed a reconstitution protocol for the uniformly ^{15}N labeled Y_2 receptor into a bicelle-like lipid structure with high yields suitable for NMR studies. Milligram quantities of target protein were expressed in *Escherichia coli* using an optimized fermentation process in defined medium yielding in over 10 mg/L medium of purified Y_2 receptor solubilized in SDS micelles. The structural integrity of the receptor molecules was strongly increased through refolding and subsequent reconstitution into phospholipid membranes. Specific ligand binding to the integrated receptor was determined using radioligand affinity assay. Further, by NMR measurement a dispersion of the ^{15}N signals comparable to native rhodopsin was shown. The efficiency of the reconstitution could also be inferred from the fact that reasonable ^{13}C NMR spectra at natural abundance could be acquired.

1. Introduction

G protein-coupled receptors (GPCRs) are essential in various signal transduction pathways and consequently represent an extremely important and promising target for pharmaceutical research. For the structure-based drug development, atomistically resolved conformational data represents a prerequisite [1], which is usually provided by X-ray diffraction or NMR spectroscopy. Although there are ~800 GPCRs found in the human genome, so far, structural data for only four full-length GPCRs is available [2–5]. In spite of the recent progress in the field, there is still a large discrepancy between the urge for highly resolved structures from basic research as well as from the pharmaceutical industry on the one hand and the available data on the other. Although the four available crystal structures of the GPCRs demonstrate the high structural homology in the class A GPCR family, it is unlikely that static views of GPCRs in non-native crystalline environments can provide all the information for the understanding of protein function of these highly dynamic molecules and trigger the rational drug development [6]. Clearly, other biophysical methods that study GPCR molecules in their membrane reconstituted need to be involved. For the best characterized GPCR – bovine rhodopsin – these methods have contributed a wealth of information, which has led to a rather comprehensive understanding of the biological function of the molecule and the entire signal transduction in the retina [7–10]. The need for studying GPCRs in natural membrane environment with particular attention to the protein dynamics has also been recognized by crystallographers [6]. Clearly, a natural lipid environment would be most suitable for such studies requiring the development of efficient reconstitution protocols for GPCRs.

In particular NMR spectroscopy is recognized as a versatile tool for the understanding of membrane protein structure and dynamics [6,11]. Especially solid-state NMR is amenable to membrane proteins in their natural environment [12–16]. But NMR measurements require large amounts of isotopically labeled, highly structured, and stable protein samples. The preparation of GPCRs in this quality as well as in sufficient quantity has represented a serious limitation for structural and dynamical studies by NMR methods so far [17].

Among the many methods for protein synthesis in various microorganisms, the recombinant expression of GPCRs in *Escherichia*

Abbreviations: CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DMPC-d_{67}, 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphatidylcholine-1,1,2,2-d_4-N,N,N-tri-methyl-d_9; DDM, n-dodecyl-β-D-maltoside; GPCRs, G protein-coupled receptors; ^{3}H-NPY, N-[propionyl-^{3}H]-pNPY; HSQC, heteronuclear single quantum coherence; IPTG, isopropylthiogalactosid; LUV, large unilamellar vesicle; MAS, magic angle spinning; NPY, neuropeptide Y; SDS, sodiumdodecylsulfate.

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coli (*E. coli*) as inclusion bodies provides a method for the production of large amounts of labeled protein at a favourable cost–yield ratio [18]. But proteins in inclusion bodies represent inactive precipitates within the cell plasma [19] and, therefore, have to be isolated, solubilized and folded *in vitro* into their native structure. Although this strategy still represents an enormous challenge, some studies have already shown that this method can produce functional GPCRs [20–24]. Recently, a first NMR structures of the membrane protein VDAC, which was refolded from inclusion bodies, has been published [25,26], which underlines the great potential of this method.

In spite of this success, the characterization of native GPCRs *in vitro* is extremely difficult, since the non-active aggregated state has a lower free energy than the fully-structured active state. Therefore, the active state has to be stabilized by amphiphilic molecules to keep the receptor in solution. Non-denaturizing detergents are suitable to refold denaturized receptors [27] or to solubilize GPCRs from cellular membranes into an active state [28–30], but they provide only a fairly unstable environment for hydrophobic proteins, since the detergent molecules are in constant exchange between the monomeric and the micellar states. In contrast, phospholipids build up very stable membrane structures, where reconstituted GPCRs can be characterized in high concentrations [31,32].

The challenge for reconstitution is to force the hydrophobic helical receptor domains out of the micelles, through the hydrophilic lipid–water interface into the hydrophobic membrane core, without losing the GPCR molecules by aggregation [33]. However, the most promising strategy to insert efficient amounts of receptor proteins into phospholipid bilayers is a detergent mediated reconstitution process [34]. Since detergents are efficient in breaking off lipid interactions, they are suitable to destabilize rigid lipid structures [35] or to solubilize them into mixed micelle structures [36], and, therefore, lower the thermodynamic energy barrier. This process is reversible and the lipid structures with integrated receptor molecules are reformed again after detergent removal below the critical micelle concentration [37].

Here, we describe the membrane reconstitution of the refolded human neuropeptide Y receptor Type 2 (Y_2 receptor) into phospholipid membranes. The Y_2 receptor is typical member of the class A GPCR family [38] with important pharmacological functions [39]. Receptor signalling is triggered by binding of the 36-amino acid peptide amide neuropeptide Y (NPY), which represents the natural ligand of the Y_2 receptor [40]. This article focuses on the expression of large amounts of labeled GPCR as well as the characterization of receptor structure and function before and after reconstitution by ligand binding and NMR measurement.

2. Materials and methods

2.1. Materials

The ligand porcine NPY (pNPY) was synthesized by automated solid-phase peptide synthesis following the Fmoc/^{t}Bu (9-fluorenylmethoxycarbonyl-tert-butyl) strategy [41]. N-[propionyl-^{3}H]-pNPY (^{3}H-NPY) was purchased from Amersham Biosciences (Buckinghamshire, UK) and Ni Agarose was obtained from Qiagen (Hilden, Germany). Stable isotopes were purchased from Euriso-Top (Saarbrücken, Germany). Trace elements and vitamins in the cultivation media were obtained from Carl Roth (Karlsruhe, Germany) and phospholipids were purchased from Otto Nordwald (Hamburg, Germany). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.2. Expression and purification

The *E. coli* strain BL21(DE3)-R3-pRARE2 (Novagen, Madison) was transformed with the cDNA of the Y_2 receptor using the vector DNA pET41b, which provides the expression of the Y_2 receptor with a C-terminal 8×His-tag to enable easy purification. To obtain sufficient amounts of the ^{15}N-labeled Y_2 receptor, the target protein was expressed in *E. coli* as inclusion bodies using a fed-batch fermentation process with defined medium.

Cells were adapted to minimal media in three stages by inoculation in the medium from the previous adaptation phase. The cultivation medium consisted of 29.2 g/L K_2HPO_4, 8.14 g/L $NaH_2PO_4\times 2H_2O$, 9.29 g/L Na_2SO_4, 1.2 g/L $MgSO_4\times 7H_2O$, 2.075 mg/L $CaCl_2\times 2H_2O$, 0.525 mg/L $CoCl_2\times 6H_2O$, 43.4 mg/L EDTA, 25.1 mg/L $FeCl_3$, 0.25 mg/L $CuSO_4\times 5H_2O$, 0.25 mg/L $MnSO_4\times H_2O$, 0.45 mg/L $ZnSO_4\times 7H_2O$, 100 mg/L thiamine hydrochloride, 34 mg/L chloramphenicol, and 100 mg/L kanamycin sulfate. The supplementation for shake flasks (*i*), fermentation batch medium (*ii*) and fermentation feed medium (*iii*) differed for glucose (*i* 5 g/L, *ii* 25 g/L, *iii* 400 g/L), natural abundance NH_4Cl (*i* 1 g/L, *ii* 0 g/L, *iii* 0 g/L), $^{15}NH_4Cl$ (*i* 0 g/L, *ii* 2 g/L, *iii* 1 g/L), natural abundance $(NH_4)_2SO_4$ (*i* 4.92 g/L, *ii* 0 g/L, *iii* 0 g/L) and $(^{15}NH_4)_2SO_4$ (*i* 0 g/L, *ii* 9.84 g/L, *iii* 4.92 g/L).

During expression, the optical density at 600 nm was measured hourly or even half hourly to provide information about cell growth. Substrate concentration was determined by using a blood glucose meter ACCU CHEK® Aviva (Roche Diagnostics, Mannheim, Germany). A calibration curve was generated using defined concentrations of glucose diluted in the cultivation medium. Cell broth glucose concentration was detected after centrifugation.

Fermentation was carried out in a BIOSTAT® C-DCU bioreactor (B. Braun Biotech, Melsungen, Germany) with MFCS/win 1.0 software for data acquisition. A preparatory culture of 25 mL medium was used to inoculate 5×200 mL medium. At an optical density (600 nm) of around 3.0 cells were pelletized by centrifugation. The cell pellet was re-suspended in 50 mL tap water and used to inoculate the 5 L batch medium. Fermentation parameters were controlled by the digital control unit including the control of the dissolved oxygen by a cascade of airflow, stirrer speed, and pure oxygen flow to 30%, the temperature (37 °C), the pH by the addition of 10% NaOH or 10% H_3PO_4 to a value of 7.0, and of the feed flow by gravimetric flow control to the set profile. Cell growth and substrate concentration were measured hourly or half hourly, respectively. The expression of the Y_2 receptor was induced by the addition of 1 mM IPTG and the cells were cultivated for additional 3 h. After cell harvesting by centrifugation at 4 °C the pellet was stored at −80 °C.

The isolation and the solubilization of the expressed inclusion bodies, as well as the purification of the Y_2 receptor out of the inclusion bodies into SDS micelles were performed as previously described [23].

2.3. Circular dichroism measurements

The Far-UV CD spectrum of the Y_2 receptor was recorded on a Jasco J-816 CD spectrometer (Jasco, Gross-Umstadt, Germany) in a wavelength from 190 to 260 nm and a continuing scanning mode, with a slit width of 1 nm and an integration time of 1 s. The sample was measured in a 0.2 mm quartz cuvette at a temperature of 20 °C and a protein concentration of 0.4 mg/mL in a buffer containing 50 mM sodium phosphate pH 8.0 and 15 mM SDS. A reference of the buffer was measured under identical conditions and subtracted from the spectrum. The secondary structure content was estimated using the CDpro package [42].

2.4. Reconstitution

To assemble the native conformation, the Y_2 receptor was folded *in vitro* by rapid dilution into a buffer containing micelles composed of the mild detergents n-dodecyl-ß-D-maltoside (DDM) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and subsequently concentrated as it is previously described [23] to a concentration of around 20 µM. Since the folded receptor in the

micellar state loses its ability to bind NPY almost completely within 3 days (data not shown), the Y_2 receptor was reconstituted in a lipid environment directly after concentrating.

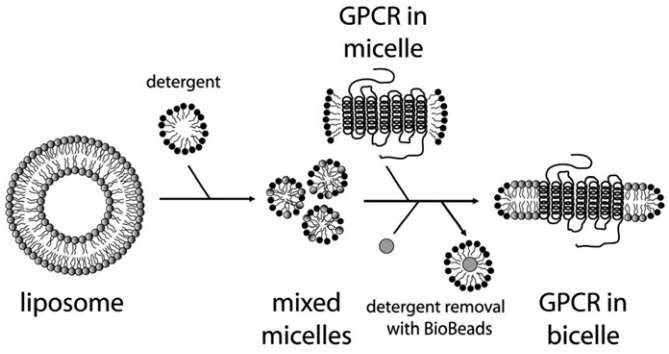
The strategy used for the reconstitution of the Y_2 receptor is shown in Scheme 1. Initially, large unilamellar vesicles (LUVs) composed of pure 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and a diameter of 100 nm were prepared [43]. These LUVs were completely solubilized with an 8-fold molar excess of the zwitterionic detergent CHAPS to small mixed micelle structures. Afterwards, the phospholipid/detergent solution was incubated with the refolded Y_2 receptor in a receptor/phospholipid 1/3 mass ratio for 1 h. Then, the detergent concentration was decreased by addition of 100 mg/mL BioBeadsSM2 (Bio-Rad Lab., München, Germany) to the solution [44]. After an incubation time of 12 h the BioBeads were removed by filtration with a sieve of 0.5 mm pore size. Finally the solution with the reconstituted receptor was dialyzed against 50 mM sodium phosphate buffer at pH 8.0 for at least 24 h to remove all glycerol. For the determination of the protein concentration of the Y_2 receptor in the phospholipid structures the Schaffner–Weissman assay was used [45]. As a standard, the Y_2 receptor in SDS micelles, prepared the same way and compared with spectrophotometrically measurements, was used.

The radioligand-binding experiment with the reconstituted Y_2 receptor was performed as it is described for the micellar Y_2 receptor [23] with a recombinant receptor concentration of around 100 nM and a ^{3}H-NPY concentration of around 10 nM.

2.5. NMR measurements

For the ^{1}H/^{15}N HSQC measurements in solution, the ^{15}N-labeled Y_2 receptor in SDS micelles was concentrated using AMICON Ultra centrifugal filter device (Millipore, Billerica, USA) with a 50 kDa cut off to a concentration of 200 µM at pH 5.7. A standard gradient HSQC experiment with pulse length of 11.93 µs and 35.30 µs for 90° pulses on the ^{1}H and ^{15}N channel, respectively, was used. The spectrum was measured on a Bruker DRX 600 NMR spectrometer at a temperature of 293 K for 20 h.

Static ^{31}P NMR spectra of the lipid phase during reconstitution were measured using a Hahn-echo pulse sequence on a Bruker DRX 600 NMR spectrometer (resonance frequency for ^{31}P 242.8 MHz). A ^{31}P 90° pulse length of 11 µs, a Hahn-echo delay of 50 µs, a spectral width of 100 kHz, and a relaxation delay of 2.5 s were applied. Continuous-wave low power proton decoupling was applied during signal acquisition. All measurements were conducted at a temperature of 20 °C.



Scheme 1. Schematic illustration for the reconstitution process of the Y_2 receptor from micelles into bilayer structures. DMPC liposomes prepared by extrusion are totally solubilized using the detergent CHAPS. Then, the mixed micelles are incubated with the refolded receptor in DDM/CHAPS. Through the reduction of the detergent concentration by adding BioBeadsSM2, the phospholipids are forced to form membrane structures covering the hydrophobic domains of the Y_2 receptors. According to the results of this study, these lipid structures could be imagined as bicelles.

For the solid-state NMR measurements, the reconstituted Y_2 receptor with a molar protein/phospholipid ratio of around 1/200 was concentrated using dialyses against a 30 wt.% PEG 20,000 solution in the dialyses buffer over several hours until the phospholipid/protein solution had a paste-like form. The MWCO was 1000. Solid-state NMR spectra were measured on a Bruker Avance 750 NMR spectrometer equipped with a double channel MAS probe at various temperatures. Standard cross-polarization experiments using a ^{1}H pulse length of 4 µs and a spin lock field on the order of 50 kHz were carried out. Standard TPPM decoupling was applied during detection with a decoupling field of 65 kHz. The MAS frequency was 5 kHz and the temperature for the measurements were −30, 1, or 30 °C

3. Results

3.1. Expression and purification

The strategy for the fermentation process was designed to generate high cell densities before induction and to produce high amounts of ^{15}N-labeled target receptor protein. In Fig. 1, the time courses of the substrate and the cell density are shown. The batch volume of 5 L supplemented with 25 g/L glucose was inoculated with a calculated amount of cells to achieve an optical density of 0.4. Cells grew with their maximum specific growth rate of 0.25 g/(gh), which represents a doubling time of 2.8 h, until total consumption of the batch glucose after 17.5 h. At this point, a feed profile aligned to the cell system was started for the growth of the cells with a specific growth rate of 0.22 g/(gh). This metered addition was reduced to a set specific growth rate of 0.14 g/(gh) within 30 min until the point of induction in that way that an estimated optical density of 45 was reached at this point. The reduction of the feed addition was performed on the one hand to avoid glucose accumulation after induction. On the other hand, it has been reported that reduced growth rates lead to higher protein production rates [46].

It is supposed that the formation of inclusion bodies reduces the toxic effect of GPCRs on the expression host [18], but this does not seem to be the case for the expression strategy presented here. The expression of the Y_2 receptor caused a strong decrease in optical density after induction due to cell lyses. This effect is perhaps due to the association of the highly hydrophobic receptor domains to the cell membranes and the formation of membrane pores, which led to the leakage of the cells [47]. Nevertheless, it was possible to produce around 13 mg of highly pure and labeled Y_2 receptor in 1 L medium (Table 1).

3.2. Characterization of the Y_2 receptor in SDS micelles

The Y_2 receptor was solubilized and purified in SDS micelles. SDS is an anionic detergent and known to denature protein structures [48]. A specific binding of NPY to its recombinant Y_2 receptor in SDS micelles was not detectable (see below). Nevertheless, the CD spectrum of this preparation, shown in Fig. 2, exhibits two minima at 208 nm and 222 nm, as well as a maximum at 192 nm, which represents the typical signature of α-helical proteins. The calculated α-helical content of the Y_2 receptor in SDS micelles was around 50%, which is equivalent to the expected content of 53% for the native structure, as it has been reported for other GPCRs [49].

Also in the solution HSQC NMR spectrum of the Y_2 receptor in SDS shown in Fig. 3 various isolated signals could be detected. The signals occupy a region, which is typical for α-helical membrane proteins [50]. Furthermore, the dispersion of the signals exceeds by far the range, which would be expected for denaturized proteins and is even comparable to other seven transmembrane helix non-GPCR membrane proteins [51]. On the other hand this dispersion of the signals is still lower than what has been achieved for active rhodopsin [52], which is also shown in Fig. 3. These results suggest that the secondary

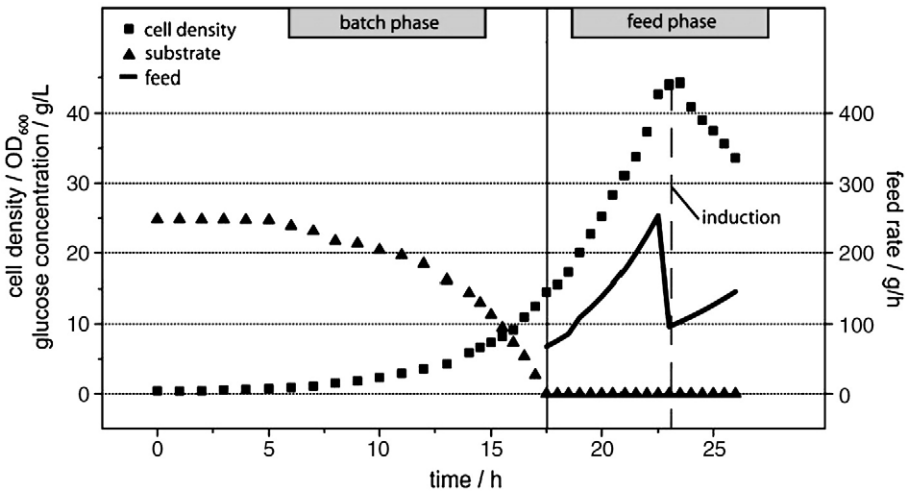


Fig. 1. Time courses of the growth and the glucose consumption of *E. coli* cells in fed-batch fermentation run on bases of defined medium for the recombinant expression of ^{15}N-labeled Y_2 receptor. After the supplemented substrate glucose was consumed, the cell growth was controlled by feed restriction in the feed phase of the process. Shortly before the induction of the target protein expression at an OD_{600} of 45 by 1 mM IPTG the specific growth rate was strongly decreased to repress glucose accumulation in the phase of expression. Almost instantly, the induction led to cell death due to the high expression level of the recombinant membrane protein. Through the addition of $^{15}NH_4Cl$ and $(^{15}NH_4)_2SO_4$ as sole nitrogen sources, ^{15}N-labeled GPCR was expressed.

structure of the Y_2 receptor is well built in SDS micelles, but the full folding of the protein to the active conformation is still lacking.

3.3. Reconstitution

As oppose to detergent micelles, lipid membranes represent much more stable structures, which are well suited for the reconstitution of membrane proteins. In our work, mixed DMPC/CHAPS lipids have been used to form the membrane environment for the Y_2 receptor. Similar mixtures of phospholipids and detergents have been identified to form bicelles [53].

To investigate the lipid phase state of the phospholipid reconstitution system for the receptor we used static ^{31}P NMR. The ^{31}P NMR spectrum of pure DMPC-LUV shown in Fig. 4A exhibits the typical line shape of lamellar liquid-crystalline phase of nearly spherical phospholipid vesicles. The chemical shift anisotropy $\Delta\sigma$ in this spectrum was determined to be 51.5 ppm. After the addition of the detergents, the line shape changes dramatically to one isotropic peak representing the signal of phospholipids in small highly mobile micelles (Fig. 4B) [54]. After adding the Y_2 receptor and the removal of the detergents using BioBeadsSM2, again an anisotropic line shape arises (Fig. 4C). This spectrum consists of an anisotropic spectrum with a maximum at ~−13 ppm and a low field shoulder superimposed with a small isotropic line at 0 ppm. Only 3% of the entire spectral intensity is represented by this isotropic peak in the spectrum. Such ^{31}P NMR line shape of DMPC/CHAPS mixtures is reminiscent of bicelle structures as investigated in detail by several groups [55–57]. If the anisotropic line shape represented a non-curved bilayer structure, a low field shoulder of ~26 ppm would have to occur; this is clearly not the case. Nevertheless, anisotropic line shapes are not the expected line for a well-oriented bicelle, but similar spectra have also been observed in the literature [58,59].

To get further insights into these lipid structures, we added 10 mM Mn^{2+} ions to the sample. Due to its paramagnetic properties, Mn^{2+} quenches all phosphorus signal, which are accessible from the aqueous phase. All signals arising from phospholipid headgroups at the inner leaflet of a closed bilayer structure would remain in the spectrum. Since there is absolutely no signal left (shown in Fig. 4D) all phospholipid headgroups are accessible to Mn^{2+} in the water. Such results would also be in agreement with bicellar structures that are oriented in the external magnetic field [55–57]. In light scattering measurements an average diameter of around 110 nm could be determined for the bicelles (data not shown).

It was not the goal of the current study to investigate the lipid phase of the reconstitution system in great detail. In addition, the word 'bicelle' refers to numerous structures that highly depend on temperature and are likely to change upon reconstitution of any membrane protein [55–57]. However, we can adhere to the statement

Table 1
Yields of biomass, inclusion bodies, and pure Y_2 receptor in different stages of preparation, calculated for 1 L fermentation volume. The calculation was done for inclusion of batch starting volume and volume increase due to feed and base addition. Biomass and inclusion bodies were weighted\purified and refolded receptors were estimated spectrophotometrically using an extinction coefficient of 54.360 M^{-1} cm^{-1} and the amount of reconstituted receptor was determined by measuring the absorption at 630 nm of precipitated, washed and with amido black dyed protein.

Yields from 1 L medium	
61 g	Biomass
5.6 g	Inclusion bodies
13.4 mg	Purified Y_2 receptor
10.9 mg	Refolded and concentrated Y_2 receptor
8.7 mg	Reconstituted Y_2 receptor

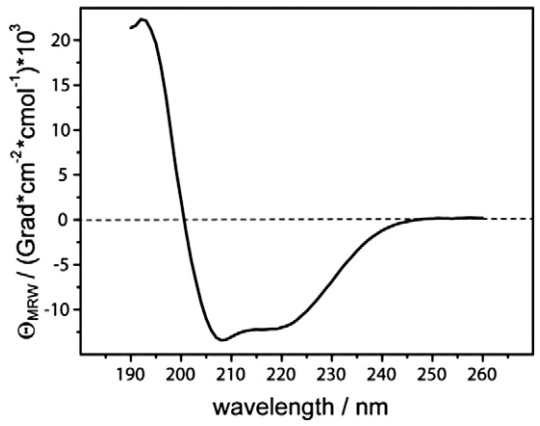


Fig. 2. Far-UV CD spectrum of the Y_2 receptor in SDS micelles. From the curve an α-helical secondary structure content of ~50% could be calculated, which corresponds to what is expected for the native Y_2 receptor. The spectrum is given as molar ellipticity values per amino acid residue.

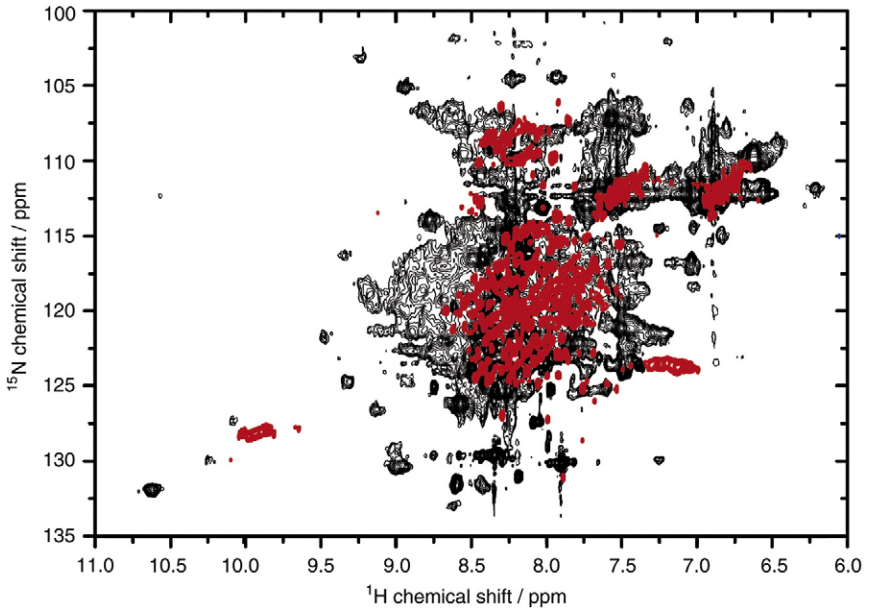


Fig. 3. Overlay of $^{1}H/^{15}N$ HSQC spectra of the Y_2 receptor in SDS micelles (red) and native rhodopsin (black), which was reproduced from literature [52]. The spectrum of the Y_2 receptor in SDS micelles was measured at 293 K with a protein concentration of 200 μM at pH 5.7. The one-dimensional projections of this spectrum are presented on the sides. The HSQC spectrum of rhodopsin shows much better signal dispersion but somewhat bigger linewidth. Compared to rhodopsin, the HSQC spectrum of the Y_2 receptor in SDS is missing signals at high chemical shifts, which are typical for intact tertiary structures.

that our reconstitution system features single layered bilayer membrane patches that partially orient in the external magnetic field.

3.4. Characterization of the Y_2 receptor in membrane environment

Using the strategy for reconstitution of the Y_2 receptors from the micellar into a lipid environment, summarized in Scheme 1, high amounts of the refolded Y_2 receptor could be reconstituted into bicelle-like lipid structures with a molar protein/phospholipid ratio of around 1/200. Less than 20% of the protein was lost by aggregation during the reconstitution process.

To assess if the functionality of the refolded Y_2 receptor was retained during reconstitution, radioligand-binding studies were carried out. In Fig. 5, the specific binding of ^{3}H-labeled NPY to receptor preparations in different stages are shown. The specific binding was calculated as the difference of the total binding and the unspecific binding divided by total binding [60]. As expected, for the Y_2 receptor in SDS micelles, no significant specific binding was detectable. In contrast, after folding and reconstitution NPY is able to bind to the receptor preparation, demonstrating that the native structure could be assembled, at least in terms of ligand binding. Additionally, the ability to specifically bind the NPY ligand could be obtained for a minimum of 12 days, showing a high long term stability of the reconstituted Y_2 receptor.

Also, the receptor in bicelles is highly structured, which can be inferred from ^{15}N solid-state NMR measurements. The dispersion of the NH signals is strongly increased through the refolding and reconstitution of the Y_2 receptor (Fig. 6A) compared to the HSQC spectrum in SDS micelles. Unfortunately, the line width under solid-state NMR conditions is much larger such that no resolved NMR lines

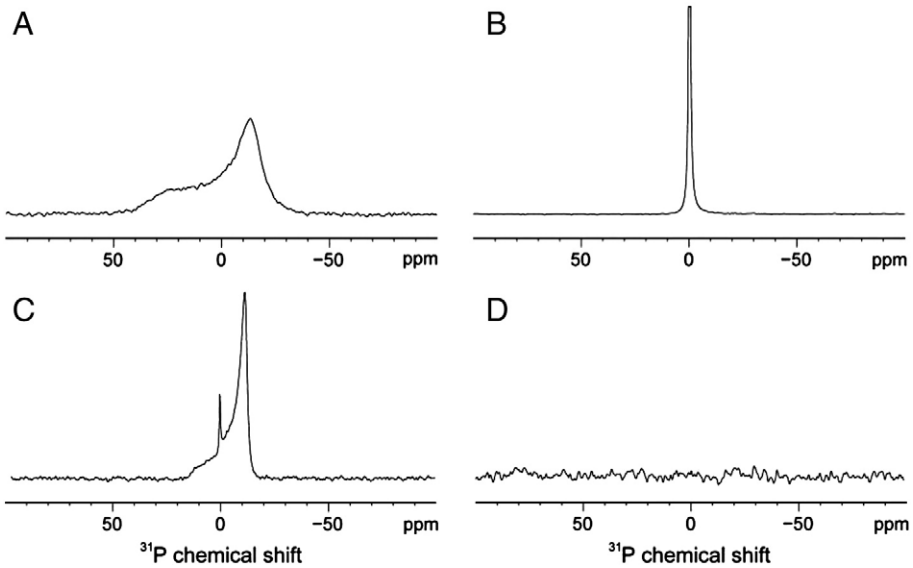


Fig. 4. Static 242.8 MHz ^{31}P NMR spectra of the phospholipid structures in different stages of the reconstitution. Shown are the ^{31}P NMR spectra of (A) pure DMPC liposomes, (B) mixed micelles after addition of the detergent (CHAPS), (C) phospholipid structures after detergent removal and (D) spectrum C after addition of 10 mM Mn^{2+}.

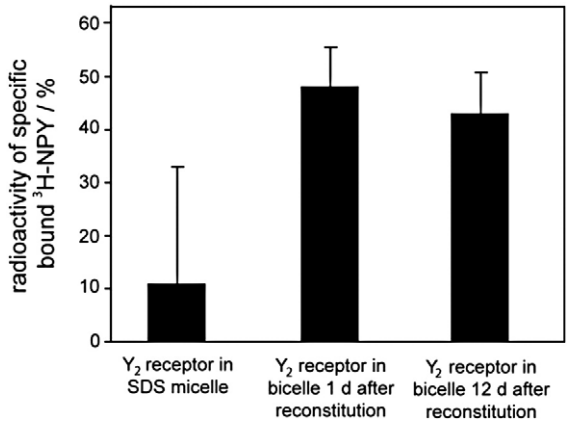


Fig. 5. Radioactivity of specifically Y_2 receptor bound ^{3}H-labeled NPY in different stages of preparation. No significant specific binding of NPY to the receptor preparation in SDS micelles was detectable. In contrast, around 50% specific binding could be obtained after refolding and reconstitution of the Y_2 receptor into bicelles. Additionally, this ability to bind NPY by the reconstituted receptor preparation was kept almost completely over at least 12 days.

can be detected [14]. It is interesting that the ^{15}N solid-state NMR lineshape closely resembles the envelope of a simulated ^{15}N NMR spectrum of bovine rhodopsin [61] (Fig. 6B), which was simulated on the basis of the crystal structure using ShiftX [62]. This indicates that the structural integrity of the Y_2 receptor in the lipid environment is much better than seen from the SDS micelles, which agrees with the radioligand assay that the reconstituted receptor is in a functional state. Thus, this reconstituted receptor system may represent a promising model for further biophysical studies.

To demonstrate the high efficiency of the reconstitution, we also recorded a natural abundance ^{13}C CPMAS NMR spectrum of the Y_2 receptor in membrane environment. Fig. 7 shows the ^{13}C NMR spectrum of Y_2 receptor in the reconstitution system at a temperature of 1 °C. This spectrum was accumulated overnight in 43008 transients. Clearly, prominent signals for the Cα and CO backbone as well as the sidechain carbons can be detected with a reasonable dispersion typical for helical proteins. Since deuterated DMPC-d_{67} was used, no significant contributions from the lipid background are observed. This demonstrates that even for non-^{13}C labeled receptor, reasonable NMR spectra can be acquired, which can further be improved by ^{13}C labeling and investigation at colder temperature [63]. ^{13}C NMR spectra of similar quality could be acquired at −30 °C within only 2048 scans.

4. Discussion

Solid-state NMR measurements of GPCRs can provide insights into the mechanisms of ligand–receptor interactions as well as structural information of the entire protein molecule in its native environment. But this method as any structural methods requires large amounts of highly structured receptors, which are reconstituted in lipid bilayers and labeled with NMR active isotopes. Therefore, the first focus of this study was to develop a fermentation strategy in minimal medium for cost effective expression of isotopically labeled GPCRs. Through extensive process optimizations (Berger et al., in preparation), at least 13 mg/L medium of fully ^{15}N-labeled Y_2 receptor could be produced and also the possibility of a ^{13}C labeling is provided by this medium composition.

Next we briefly studied the solubilized receptor molecules in SDS micelles. The data indicate, that the ionic detergent SDS is not as denaturizing as one would expect from literature [48]. Clearly, the Y_2 receptor in SDS does not exhibit the full structural integrity, which is particularly indicated not only by the lack of specific NPY ligand binding but also by the absence of the downfield signals in the NMR spectra. Nevertheless, the NMR spectra of the Y_2 receptor in SDS indicate large amounts of α-helical structures in agreement with data, for instance on the KcsA channel [64]. This suggests that in particular secondary structure elements are formed as also seen in the CD spectra but that the full tertiary structure of the protein not formed under these conditions. Such structures would exhibit more signal dispersion as particularly tertiary contacts between aromatic amino acid sidechains and the backbone signals result in ring current effects that further disperse the signals in the HSQC spectra. It can be concluded, that some domains of the molecule are already aligned, while others are not due to the lack of electrostatic interactions and the fact that different helices of the receptor are kept in different micelles, as it is indicated in Scheme 2A. Work by the Zerbe group has also shown that domains of GPCRs show correct tertiary contacts in LMPG micelles as observed by long range NOEs [50]. More and more data accumulates stating that the correct choice of detergents is most crucial for the stable preparation of membrane proteins [65].

The alignment of all protein domains into a correctly folded and functional structure (Scheme 2B) is achieved through a detergent exchange from SDS to a DDM/CHAPS micelle system, as we determined earlier [23]. The formation of electrostatic interactions between the helices of the Y_2 receptor seem to be the driving force for correct folding, which are facilitated in these non-/zwitter-ionic micelles as long as the proportion of CHAPS is not too high.

Nevertheless, detergent micelles are rather unstable objects, which show constant exchange between monomeric detergents and

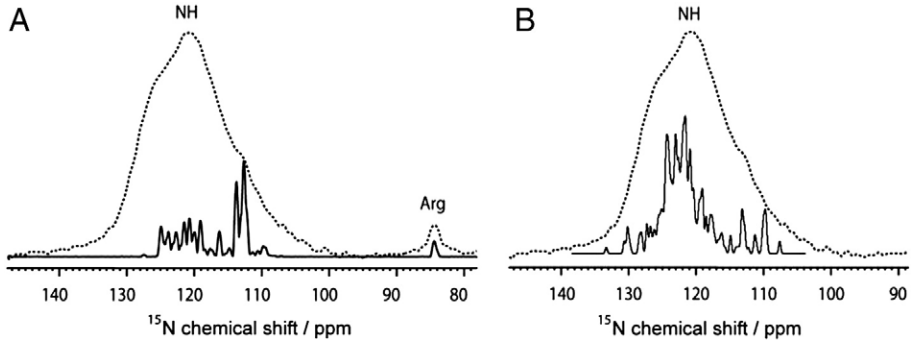


Fig. 6. Comparison of the ^{15}N CPMAS NMR spectrum of the reconstituted Y_2 receptor (dotted line) with the ^{15}N projection of the ^{1}H/^{15}N HSQC spectra of (A) the Y_2 receptor in SDS micelles and (B) a simulated spectrum of rhodopsin [61]. The ^{15}N signals of the reconstituted Y_2 receptor show a much better dispersion than in SDS micelles before folding. This dispersion of the folded and reconstituted recombinant Y_2 receptor is comparable to the signal dispersion of native rhodopsin, suggesting an equivalent structural variety. For the ^{15}N NMR spectrum simulation the theoretical chemical shifts were calculated from the PDB file using the SHIFTX program [62]. The signals were simulated as Gaussian lines with a width of 12 Hz.

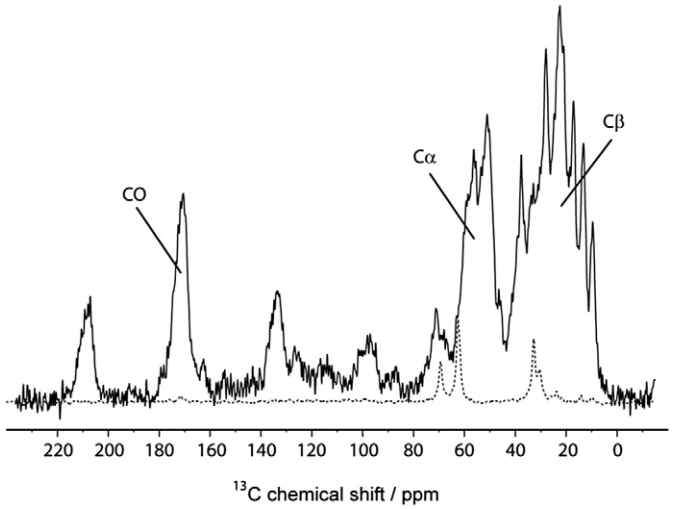
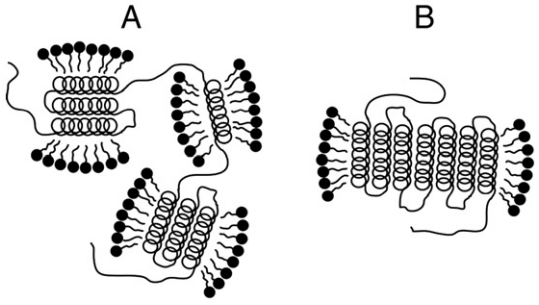


Fig. 7. ^{13}C CPMAS NMR spectra of reconstituted Y_2 receptors in DMPC membranes. The signals of the DMPC-d_{67} are shown with the dotted line. The measurement was performed at a temperature of 274 K and with a receptor/phospholipid ratio of around 1/200.

the entire micelle and temporarily expose also the hydrophobic interior to the aqueous environment through the large amplitude motions [27,37]. In contrast, phospholipid membranes are much more robust entities, which also represent the natural environment of membrane proteins. In particular, proteins sense the membrane environment and adapt to the specific physicochemical properties of the bilayer. Thus, parameters such as the membrane thickness or the phase state, for instance encountered in a lipid raft or a liquid-crystalline membrane domain, may have a tremendous impact on the structure of protein segments or the tilt of a protein in the membrane and many other parameters [66–68]. Thus, the choice of the host membrane for the reconstitution of a respective membrane protein may be as important as the correct detergent for solution NMR studies.

Prerequisite for reconstitution of proteins into membranes for solid-state NMR measurements is a dense incorporation, since high protein concentration is required for sufficient sensitivity. This could not be obtained by directly assembling of the Y_2 receptor into preformed liposomes, because the receptor molecules in the micelles are too unstable to overcome the thermodynamic barrier on the lipid–water interface. Instead, the phospholipid chains have to associate with the hydrophobic domains of the receptor, which is a thermodynamically favourable process. To achieve this, phospholipids and detergents were co-solubilized with the receptor and the detergents



Scheme 2. Theoretical tertiary structure of the helical receptor domains in (A) SDS micelles and in (B) non denaturating micelles after *in vitro* folding. After solubilization and purification in SDS, the structure of the Y_2 receptor features a large α-helical content and some hydrophobic domains may already be in the correct tertiary structure in different micelles. The native tertiary structure is obtained by *in vitro* folding of the Y_2 receptor in DDM/CHAPS micelles or reconstitution into membrane systems.

were subsequently removed. This procedure and in particular the use of the phospholipid DMPC and the detergent CHAPS resulted in planar membrane structures that were reminiscent of bicelles. Indeed, it has been reported that DMPC and the detergent CHAPSO formed bicelles [53]. Although the morphology of the lipid structures used here was not analysed in great detail, bicellar structures represent some advantages for the investigation of GPCRs, in particular the accessibility of both sides of the molecule, which would allow to study ligand binding on the N-terminal ('extracellular') side and subsequent G protein activation on the C-terminal ('intracellular') side [69]. Also for solid-state NMR, bicelles represent a good membrane system as they feature a favourable protein to lipid ratio and most of the excess water can be removed as done in the current study.

Using ^{15}N CPMAS NMR measurements, we studied the signal dispersion of the receptor backbone, which is related to the structural integrity of the molecules. Compared to the micellar state in SDS, a much better dispersion of the backbone ^{15}N signals could be observed, which was comparable to that of functional rhodopsin. Although no site resolution was observed under these conditions, the ^{15}N signals show a much broader dispersion than in the SDS micelles. This is confirmed by the ^{13}C CPMAS NMR spectrum that showed a typical dispersion of the Cα signals as known from well-folded proteins. Since the signal dispersion represents the structural integrity of proteins [70] and rhodopsin is suitable as basic structural model for class A GPCRs [71], it can be suggested, that the Y_2 receptor has a native-like conformation in the reconstitution system, which agrees with the radioactivity measurements. Although the exact portion of native receptors was not determined, the high signal-to-noise ratios in the NMR spectra indicate homogenous preparations with mainly highly structured receptor molecules. Most likely, this structured fraction refers to the native fraction, to which the ligand is able to bind as shown in the radioligand-binding assay. After refolding the preparation presented a mix of functional and non-functional molecules [23]. However, reconstitution into the native membrane environment may force the receptor into a native structure because of the specificity of the lateral stress profile in membranes and the particularities of the lipid–water interface [72,73]. It is conceivable that the protein, which is lost during reconstitution is the wrongly folded conformation as it does not 'fit' into the membrane and natively structured proteins represent the more stable fraction [74]. Further, not fully-structured folding intermediates may become natively structured in the phospholipid environment by the lateral pressure of the bilayer. Such folding effects triggered by reconstitution are already described for the outer membrane proteins of *E. coli* [75] and may represent the most important driving force for the successful reconstitution of GPCRs as well. While the determination of complete structures of GPCRs and other membrane proteins are by far not a standard procedure as of now, with the techniques demonstrated here, a number of specific biophysical questions can already be well addressed. Further success of the techniques will rely on the resolution and sensitivity of the solid-state NMR technique for structure determination.

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