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Review

# Recombinant G protein-coupled receptors from expression to renaturation: a challenge towards structure

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**Abstract.** G protein-coupled receptors (GPCRS) represent a class of integral membrane proteins involved in many biological processes and pathologies. Fifty percent of all modern drugs and almost 25% of the top 200 best-selling drugs are estimated to target GPCRs. Despite these crucial biological implications, very little is known, at atomic resolution, about the detailed molecular mechanisms by which these membrane proteins are able to recognize their extra-cellular stimuli and transmit the associated messages. Obviously, our understanding of GPCR functioning would be greatly facilitated by the availability of high-resolution three-dimensional (3D) structural data. However, expression, solubilization and purification of these membrane proteins are not easy to achieve, and at present, only one 3D structure has been determined, that of bovine rhodopsin. This review presents and compares the different successful strategies which have been applied to solubilize and purify recombinant GPCRs in the perspective of structural biology experiments.

**Keywords.** Recombinant, membrane protein, GPCR, solubilization, purification, structure.

## Introduction

G protein-coupled receptors (GPCRs) are integral membrane proteins composed of seven transmembrane spanning domains [1]. Among the numerous vital functions subserved by GPCRs are those of three of the five senses: vision, taste and smell. These cell surface receptors mediate signal transduction of numerous hormones, neurotransmitters and drugs across the plasma membrane. Fifty percent of all modern drugs and almost 25% of the top 200 best-selling drugs are estimated to target GPCRs [2]. GPCR-targeting drugs are used to treat a wide variety of pathologies including cardiovascular and gastrointestinal diseases, central nervous system and immune disorders and cancer [2]. Despite these crucial biological implications, very little is known, at atomic resolution, about the detailed molecular mechanisms by which these membrane proteins are able to recognize their extracellular stimuli and transmit the associated messages through endogenous heterotrimeric G proteins. The association of the receptor with its cognate ligand is expected to give rise to conformational and structural changes of the protein, but, for the vast majority of GPCRs, no direct proof has yet been found. Obviously, our understanding of GPCR functioning would be greatly facilitated by the availability of high-resolution three-dimensional (3D) structural data. In this perspective, the two main techniques available providing 3D structural information at atomic resolution, namely nuclear magnetic resonance (NMR) and X-ray crystallography (Table 1), both require large amounts of purified protein. Consequently, at present, the only GPCR 3D structure which has been determined is that of the vision receptor rhodopsin, a GPCR which is naturally present nearly pure in retina, and in

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high quantities [3]. In contrast to rhodopsin, other GPCRs are naturally weakly expressed and their direct purification from natural sources would be unfeasible in the perspective of structural biology experiments. Thus, the heterologous over-expression of GPCRs is a necessary step, which, as discussed elsewhere [4], is not easy to overcome. In addition, once this first step has been achieved, the recombinant protein has to be extracted from the host organism (solubilized) and purified. At this stage, two strategies may be followed. The first one preserves the receptor functionality during the solubilization and purification steps. In this manner, the availability of high amounts of receptor protein has allowed the determination of the receptor-bound conformations of pituary-adenylate-cyclase-activating polypeptide [5], glutamate [6], muscarine [7] and neurotensin [8]. The second strategy is focused on the refolding of an initially inactive and unfolded form of the receptor. Kiefer et al. [9, 10] were the first to demonstrate the validity of this approach for an olfactory receptor over-expressed in *Escherichia coli.* More recently, Baneres et al. [11–13] were able to produce the human leukotriene B4 receptor, $BLT_1$ and the 5-$HT_4$-hydroxytryptamine receptor in *E. coli* and to refold them from inclusion bodies with yields that were sufficient for the structural characterization of these receptors in solution. Whatever the strategy employed, a large number of detergents and/or chaotropic agents, purification strategies, reconstitution and/or crystallization trials can be foreseen (Table 1), so that the preliminary work to any structural study is lengthy. The objective of this review is to present and compare the different successful strategies that have been applied to solubilize and purify recombinant GPCRs.

## The solubilization step

### Principle and objectives

GPCRs naturally function in a membrane environment mainly constituted of proteins, phospholipids and cholesterol. The functionality of GPCRs embedded within this environment is essentially affected by the physical and chemical properties of the surrounding membranes. When a GPCR is expressed as a recombinant protein, it can follow the host cell membrane protein trafficking pathway to finally reach the plasma membrane. Alternatively, the over-expression of foreign and recombinant proteins can also induce the formation of misfolded and aggregated forms of proteins named inclusion bodies when this phenomenon happens in *E. coli* [14]. Whatever the initial state of the expressed protein, it needs to be solubilized, purified, concentrated and frequently reconstituted in a lipid environment before structural biology experiments. The lipid environment surrounding over-expressed GPCRs and the occurrence of misfolded and aggregated forms imply that before any purification attempt, the solubilization of the protein has to be achieved (Table 1). The objective of the solubilization step is to extract, using detergents and/or chaotropic agents, the membrane protein from its initial environment. During this step, the hydrophobic membrane protein will pass either from a lipid environment to a detergent micelle environment or from an aggregated form to a strong detergent- [e.g. sodium dodecyl sulfate (SDS)] or strong denaturant- (e.g. urea, guanidine-HCl) solubilized form.

### Solubilization with detergents

The ability of detergents to extract integral membrane proteins such as GPCRs from biological membranes relies, in general, on their ability to solubilize membrane lipids [15]. Detergents are compounds with amphipathic properties with the occurrence, in the same molecule, of a polar head group and a hydrophobic tail. Detergents are classified into three main categories according to their structures. Ionic detergents contain either a head group with a net charge that can be cationic (e.g. cetyl-trimethyl-ammonium bromide) or anionic (e.g. SDS) together with a hydrophobic chain, or a polar and an apolar face, instead of a well-defined head group, in the case of bile acid salts

**Table 1.** A synthetic flowchart showing the main steps and multiple choices involved in the expression of recombinant GPCR in the perspective of structural biology.

| | |
|---|---|
| **Recombinant GPCR expression** | |
| *Eukaryote* | *Prokaryote* |
| Insect cells | *Escherichia coli* |
| Mammalian cells | |
| Yeast | |
| **Solubilization of membranes or inclusion bodies** | |
| *Detergent* | *Chaotropic agent* |
| Cationic | Urea |
| Anionic | Guanidine |
| Zwitterionic | |
| **Purification** | |
| *Intrinsic properties* | *Specific tags* |
| Ion exchange | Immobilized metal affinity |
| Gel filtration | Biotin-streptavidin affinity |
| Lectin affinity | Large-tag affinity |
| | Immunoaffinity |
| | Ligand-based affinity |
| **Renaturation** | |
| Artificial membranes (bilayers) | |
| Detergent (micelles, cubic phases) | |
| **Structure at atomic resolution** | |
| NMR | |
| Cryo-electron microscopy | |
| X-ray crystallography | |

(e.g. sodium cholate). Non-ionic detergents contain uncharged hydrophilic head groups of, in general, either polyoxyethylene or glycosidic groups [e.g. Triton X-100, n-dodecyl-$\beta$-D-maltoside (DM)]. Zwitterionic detergents are composed of chemical groups bearing anionic and cationic net charges (e.g. lauryl-dimethyl-amine-N-oxide) [16–18].

The solubilization of protein-containing membranes can be described using a three-stage model [15]. In stage I, detergent is taken up by the membrane phase without solubilizing it. In stage II, detergent-saturated membranes coexist with saturated mixed-micelles whereas in stage III, membrane components are fully solubilized by incorporation into mixed micelles. Together with the removal by the detergent of a substantial part of the lipids, the hydrophobic membrane-embedded region of the membrane proteins, including some lipids, becomes enwrapped in a layer of protective detergent coating. At this stage, the membrane protein can be considered to be in a solubilized state, unless extensive protein-protein contacts prevent or retard dissociation of the protein units. Regardless of the existence of specific protein contacts, removal of all, or virtually all, lipids is usually required to ensure solubilization at the protomeric level in order to avoid non-specific protein contacts, mediated by lipids. On the other hand, if the objective is to maintain protein function in the detergent solubilized state it may be unwise to carry delipidation and deaggregation of oligomers too far [15].

**Solubilization with chaotropic agents**

Urea and guanidine-HCl are small chaotropic agents able to increase sample solubility and to minimize protein aggregation by decreasing hydrogen bonds and hydrophobic interactions. In the case of aggregated proteins found in inclusion bodies, 8 M urea and 6 M guanidine concentrations are usually used to solubilize proteins in a denatured state. Urea-containing buffers should not be heated in the presence of proteins because carbamylation will occur on amino groups (N terminus and lysine) due to the creation of isocyanates [19].

**Criteria for the choice of solubilizing agents**

Two major criteria can drive the researcher in his or her choices. The first one is the compatibility of the detergent used with the purification strategy chosen and the wish, or not, to preserve the functionality of the receptor during the solubilization and purification steps. Non-ionic detergents are generally considered to be mild and relatively non-denaturing, as they break lipid-lipid interactions and lipid-protein interactions rather than protein-protein interactions [16]. This allows many membrane proteins to be solubilized in non-ionic detergents without affecting the structural features of the protein and explains the high percentage of GPCRs solubilized with non-ionic detergents such as DM and digitonin (Table 2). Among ionic detergents, SDS is extremely effective in the solubilization of membrane proteins but is also known to be denaturing. Some membrane proteins can be renatured from SDS by transferring the protein to a renaturing detergent or lipid environment [20–23]. Zwitterionic detergents combine the properties of ionic and non-ionic detergents and are in general more deactivating than non-ionic detergents. The second criterion is the facility with which the detergent employed can subsequently be removed [detergents with a high critical micellar concentration (CMC) will be easier to eliminate], or its compatibility with crystallization trials. Nevertheless, reconstitution experiments can use a wide panel of detergents since detergents with a low CMC can be removed efficiently (and over a short period of time) using polystyrene beads [24]. The establishment of appropriate solubilization conditions still seems to be totally empirical [16]. Indeed, considerable differences in behavior exist from one membrane protein to another, from one heterologous expression system to another. Furthermore, the solubilization step is influenced by numerous parameters such as buffer composition, initial protein concentration, detergent concentration, temperature, pH, salt concentration, presence of ligand and addition of osmoprotectants such as glycerol.

**Quantification of the yield of solubilization**

The efficiency of each solubilizing agent can be quantified and this is generally performed by determining the receptor protein content in the solubilized sample before and after a centrifugation step at 100,000 g, using a method that specifically follows the GPCR. A method frequently used consists in employing radioactive ligands that specifically bind to the receptor [25, 26]. Despite its efficiency, this technique detects only the functional fraction of the solubilized receptor. Furthermore, in the presence of a solubilizing agent, numerous proteins tend to lose their ability to bind ligands. If we except harsh detergents such as SDS or N-lauroyl sarcosine (NLS), it is difficult to predict which detergent will be suitable for solubilization. Some methods such as immunoblot quantification [27, 28] or fluorescence measurements using green fluorescent protein (GFP)-tagged receptors allow quantification of the total yield of solubilization [29–32]. In the latter experiments, two different GPCRs were expressed in the same host, *Pichia pastoris*. The $ET_B$-endothelin receptor was solubilized in DM with a 30% solubilization yield and further purified on a nickel column [29]. Although DM was not the best solubilizing detergent, it was chosen because the authors argue that it was already used for successful crystallization of membrane proteins. In contrast, DM was unable to solubilize the $\mu$-

**Table 2.** Comparative study of solubilized and purified GPCRs.

| | | Solubilization | | Purification | | | Reconstitution | Reference |
|---|---|---|---|---|---|---|---|---|
| Receptor | Expression system | Expression level | Detergent/ chaotrope | Tags | Purification steps | Pure | Lipids/detergents | |
| h$\beta_2$ | *Sf9* | 5–20 pmol/mg | 0.25% DM | KT3 Ct | 1: KT3 immunoaff.<br>2: alprenolol aff. chrom. | 1–4 nmol/l | – | 66 |
| h$\beta_2$ | *Sf9* | 2.5 pmol/mg | 1% DM | 6his Ct<br>FLAG Nt | 1: $Ni^{2+}$ aff. chrom.<br>2: FLAG immunoaff.<br>3: alprenolol aff. chrom. | 5 nmol/l | – | 62 |
| h$\beta_2$ | *Sf9* | 12–19 pmol/mg | 1% digitonin | none | alprenolol aff. chrom. | ND | – | 49 |
| h$\beta_2$ | *Sf9* | ND | 1% DM | 6his Ct<br>FLAG Nt | 1: $Ni^{2+}$ aff. chrom.<br>2: alprenolol aff. chrom. | 5 nmol/l | – | 65 |
| t$\beta_2$ | *Sf9* | 2 nmol/l | 1% digitonin<br>0.2% Na cholate | none | 1: alprenolol aff. chrom.<br>2: heparin gel<br>3: gel filtration | 0.15 nmol/l | POPE/brain PS | 46 |
| t$\beta_2$ | *Sf9/Tni* | 470 pmol/l<br>7.5 mg/l | 2% DM | 6his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: alprenolol aff. chrom. | 2.5 mg/l | – | 92 |
| h$\beta_2$ | *Sf9* | 7 pmol/mg | 0.25% DM | none | lentil lectin-agarose | ND | soybean PC | 93 |
| h$\beta_2$ | *E. coli* | 6 pmol/mg | 0.5% DC sucrose | MBP Nt<br>6his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: amylose gel | ND | asolectin | 64 |
| h$\beta_2$R | *Sf9* | 17 pmol/mg | 1% DC sucrose | MBP Nt<br>6his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: amylose or alprenolol | ND | PC | 64 |
| h$\alpha_{1A,B,D}$ | HEK293 | 0.15–3.6 pmol/mg | 2% DM | FLAG Nt<br>6his Nt | 1: immunoaff. FLAG<br>2: $Ni^{2+}$ aff. chrom. | ND | – | 77 |
| h$\alpha_{2C}$ | *S. cer.* | 25 pmol/mg | 0.5% SML | none | immunoaff. chrom. | ND | PC | 39 |
| $rNTS_1$ | *E. coli* | 10–15 pmol/mg | CHAPS/CHS/DM<br>0.6/0.12/1% | Bio tag/6his/<br>FLAG/Strep Ct | 1: monomeric avidin<br>2: gel filtration<br>3: NTR aff. chrom.<br>or $Ni^{2+}$ aff. chrom. | ND | – | 38 |
| $rNTS_1$ | *E. coli* | 7–13 pmol/mg | CHAPS/CHS/DM<br>0.6/0.12/1% | 6his/FLAG<br>10his | $Ni^{2+}$ aff. chrom. | ND | – | 58 |
| $rNTS_1$ | *E. coli* | 0.45 mg/l | CHAPS/CHS/DM<br>0.1/0.02/0.02% | 10his | $Ni^{2+}$ aff. chrom. | 0.21 mg/l | | 94 |

**Table 2.** (Continued).

| | | Solubilization | | Purification | | | Reconstitution | Reference |
|---|---|---|---|---|---|---|---|---|
| Receptor | Expression system | Expression level | Detergent/ chaotrope | Tags | Purification steps | Pure | Lipids/detergents | |
| $hH_1$ | *Sf9* | 4–7 mg/l | 1% DM | 10 his | $Ni^{2+}$ aff. chrom. | 3–4 mg/l | asolectine | 28 |
| $hA_1$ | CHO-K1 | 30 pmol/mg | 2% digitonin | FLAG Nt<br>6his Nt | 1: FLAG immunoaff.<br>2: aff. chrom. $Ni^{2+}$ | ND | – | 78 |
| $hA_{2A}$ | *E. coli* | 10–20 nmol/l | DM/CHS<br>1/0.2% | 10his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: ligand aff. chrom.<br>3: cation exchange chrom. | 1.5 mg/100g wet cells | – | 42 |
| hTSHR | *E. coli* | ND | 1% Triton X-100 | 6his Nt | $Ni^{2+}$ aff. chrom. | 2.5 mg/l | – | 95 |
| $hPTH_1$ | Cos7 | 240 pmol/mg | 0.25% DM | Rho tag Ct | 1 D4 immunoaff. | 100 µg<br>20×15 cm plates | POPC/PE/DMPG<br>6/3/1 | 75 |
| hPTHR1 | HEK293 | $2.8\times10^6$ receptors/cell | 0.25% DM | Rho tag Ct | 1 D4 immunoaff. | 37.5 µg/l | – | 96 |
| pLH/CGR | L cells | ND | 1.2% Triton | – | immunoaff. | ND | – | 68 |
| hSecretin | *Sf9* | 830 pmol/mg | 10% DOC | HA | 1: $NH_4(SO_2)_2$ precipitation<br>2: gel filtration<br>3: immunoaff. | 1.4 mg/l | – | 97 |
| $hD_1$ | *S. cer.* | 0.13 pmol/mg | 4% DM | 6his Ct<br>FLAG Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: FLAG immunoaff. | 0.02 mg/l | Soybean lipids | 61 |
| $mD_1$ | NS20Y | 1–2 pmol/mg | 2% DM | 6his Nt<br>GFP Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: immunoprecipitation anti-GFP | ND | – | 81 |
| $hD_2$ | *P. pas* | 3–13 pmol/mg | DM/CHS<br>1/0.4% | 6his Nt<br>FLAG Nt | 1: $Ni^{2+}$ aff. chrom.<br>2: FLAG immunoaff. | ND | – | 26 |
| h$\delta$ | CHO | ND | 30 mM OG<br>or 1% DM | cmyc Ct<br>6his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: ligand aff. chrom. | ND | egg PC/POPG<br>75/25 | 98 |
| m$\delta$ | HEK293 | 10 pmol/mg | 1% DM | 6his Ct<br>FLAG Nt | 1: gel filtration<br>2: lectin aff. chrom.<br>3: $Ni^{2+}$aff. chrom.<br>4: FLAG immunoaff. | 10 pmol/mg | – | 47 |
| m$\mu$ | HEK293 | 10 pmol/mg | 1% DM | 6his Ct<br>FLAG Nt | 1: lectin aff. chrom.<br>2: gel filtration<br>3: $Ni^{2+}$aff. chrom.<br>4: FLAG immunoaff. | ND | – | 48 |

**Table 2.** (Continued).

| | | Solubilization | | Purification | | | Reconstitution | Reference |
|---|---|---|---|---|---|---|---|---|
| Receptor | Expression system | Expression level | Detergent/ chaotrope | Tags | Purification steps | Pure | Lipids/detergents | |
| hμ | *P. pas* | 10–30 mg/l | 8 M urea<br>0.1% SDS | 6his Ct<br>cmyc Ct<br>EGFP Nt | $Ni^{2+}$ aff. chrom. | 12 mg/l | – | 30 |
| $hCB_1$ | *P. pas* | 3.6 pmol/mg | 1% FOS12 | FLAG Nt<br>cmyc Ct<br>6his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: FLAG aff. chrom. | ND | – | 99 |
| $hCB_2$ | *P. pas* | ND | 1% DM | cmyc Ct<br>6his Ct | $Ni^{2+}$ aff. chrom. | ND | – | 100 |
| $hCB_2$ | *Sf21* | 9.3 pmol/mg | 1.5% DM<br>8 M urea | 6his Ct | $Co^{2+}$ aff. chrom. | ND | – | 101 |
| $hCB_1$ | *Sf21* | 5.4 pmol/mg | 1.5% DM<br>8 M urea | 6his Ct | $Co^{2+}$ aff. chrom. | 0.7 nmol/l | – | 102 |
| $hCB_2$ | *E. coli* | 13 pmol/mg<br>(1–2 mg/l) | DM/CHAPS/CHS<br>1/0.5/0.1% | MBP Nt<br>TrxA Ct<br>10his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: anion exchange chrom. | 1 mg/l | DPC<br>or SOPC | 103 |
| OR5 | *E. coli* | 10%<br>total protein | 1.25% NLS<br>0.2% digitonin | 6his Ct<br>GST | 2×$Ni^{2+}$ aff. chrom. | 20 mg/l | PC/PG<br>4/1 | 9 |
| rOR4<br>hOR17–4 | *Sf9* | ND | 1.5% lyso PC | FLAG Nt<br>6his Nt | 1: $Ni^{2+}$ aff. chrom.<br>2: cation exchange chrom. | ND | DMPC/CHS/DPPS/DPPE<br>54/35/10/1 | 27 |
| $pM_2$ | CHO | 117 nmol/<br>30 ml packed CHO | 1% digitonin<br>0.2% cholate | – | 1: WGA agarose<br>2: hydroxyapatite<br>3: ABT sepharose | 2 mg/30 ml<br>packed CHO | PC/PS/CHS<br>1/1/0.1 | 85 |
| $hM_2$ | *Sf9* | 10.8 nmol/l | 1% digitonin<br>0.5% cholate | 6his Ct | $Co^{2+}$ aff. chrom.<br>or ligand aff. chrom. | 4.4 nmol/l | POPE/brain PS | 63 |
| $hM_1$ | *Sf9* | 2 nmol/l | 1% digitonin<br>0.1% cholate | –<br>– | 1: ABT sepharose<br>2: hydroxyapatite | 0.3 nmol/l | POPE/brain PS | 46 |
| $hM_2$ | *Sf9* | 10–15 nmol/l | 1% digitonin<br>0.1% cholate | –<br>– | 1: ABT sepharose<br>2: hydroxyapatite | 4.5–7 nmol/l | POPE/brain PS | 46 |
| $hM_2$ | *Sf9* | – | 0.86% digitonin<br>0.17% cholate | FLAG<br>c-myc | 1: DEAE sepharose<br>2: ABT sepharose<br>3: hydroxyapatite<br>or c-myc/FLAG<br>immunoaff. | ND | – | 43 |

**Table 2.** (Continued).

| Receptor | Expression system | Solubilization | | Purification | | | Reconstitution | Reference |
|---|---|---|---|---|---|---|---|---|
| | | Expression level | Detergent/ chaotrope | Tags | Purification steps | Pure | Lipids/detergents | |
| bRho | Cos-1 | 5–10 µg/ $10^7$ cells | 1% CHAPS | – | immunoaff. chrom. | 5 µg/$10^7$ cells | – | 71 |
| bRho | *Sf9* | 4 mg/l | 20 mM CHAPS | 6his Ct | $Ni^{2+}$ aff. chrom. | 0.5–1 mg/l | retina lipids | 104 |
| bRho | HEK293S | 10 mg/l | 2% NG | – | 1D4 immunoaff. | 9 mg/l | – | 72, 73 |
| bRho | *S. cer* | 2 mg/l | 1% DM | – | 1D4 immunoaff. | 40–80 µg/l | – | 74 |
| $hmGlu_6$ | COS-7 | ND | 1% CHAPS | rho tag | 1D4 immunoaff. | ND | asolectin | 76 |
| DmmGlu | *D. m.* | 10 pmol/mg | 3% FC-12 | – | immunoaff. chrom. | 170 µg/g fly head | PE/PC/PA/Erg 55/20/10/15 | 69 |
| DmmGlu | *Sf9* | 11 pmol/mg | 7.5 mM FC-12 | – | immunoaff. chrom. | ND | – | 70 |
| hCCR5 | Cf2Th | ND | 1% Cymal 5 | rho tag Ct | 1D4 immunoaff. | ND | + | 33 |
| hCCR5 | CHO | ND | 1% Triton X-100 | cmyc Ct<br>6his Ct | $Ni^{2+}$ aff. chrom. | ND | PC/PE/SM/CHS 56/28/8/8 | 105 |
| hCXCR4 | Cf2Th | ND | 1% CHAPSO | rho tag Ct | 1D4 immunoaff. | ND | POPC/POPE/DOP 6/3/1 | 36 |
| hCCR9 | m pre-B cells | 0.5 mg/l | 2% DM<br>0.05% CHS | HA Nt<br>10his Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: $Co^{2+}$ aff. chrom. | ND | – | 106 |
| hNK1 | *Sf21* | ND | CHAPS/OG<br>0.6: 0.05% | 12his Ct | 1: $Ni^{2+}$ aff Chrom<br>2: gel filtration | ND | DOPC/CHS/LPC 2/1/1<br>or *Sf9* membranes | 25 |
| $rNK_1$ | *Sf9* | 1–2 pmol/mg | 10mM CHAPS | – | lentil lectin-agarose | ND | soybean PC | 93 |
| hTP | Cos7 | 1 nmol/10×15cm dishes | 10 mM CHAPS | 6his Ct | $Ni^{2+}$ aff. chrom. | 0.36 nmol/10×15cm dishes | – | 59 |
| Ste2<br>*S. cer* | *S. cer* | 350 pmol/mg | 1% DM | FLAG Ct<br>6his Ct | $Ni^{2+}$ aff. chrom. | 50 µg/l | POPC/POPG 60/40 | 60 |
| Ste2<br>*S. cer* | HEK 293 | – | 1% DM | hFc Nt<br>GFP Ct<br>or | protein A aff. chrom. | 2 mg/l | – | 107 |
| | | | | Strep tag Ct<br>8his Ct | streptavidin aff. chrom.<br>$Co^{2+}$ aff. chrom. | 1 mg/l | – | |

**Table 2.** (Continued).

| | | Solubilization | | Purification | | | Reconstitution | Reference |
|---|---|---|---|---|---|---|---|---|
| Receptor | Expression system | Expression level | Detergent/ chaotrope | Tags | Purification steps | Pure | Lipids/detergents | |
| Ste3<br>*S. cer* | HEK 293 | – | 1% DM | Strep tag Ct<br>8his Ct | streptavidin aff. chrom.<br>$Co^{2+}$ aff. chrom. | 0.5 mg/l | – | 107 |
| $hPAC_1$ | *Sf9* | 50–150 pmol/mg | 1% digitonin | – | 1: avidin aff. chrom.<br>2: lentil lectin-agarose | 250 µg/l | bovine brain extract | 51 |
| $hP2Y_{12}$ | *Sf9* | ND | 1% digitonin | 6his Nt | 1: $Ni^{2+}$ aff. chrom.<br>2: anion exchange chrom. | 7–13 µg/l | PE/PS/CHS<br>20/13/1 | 44 |
| $m5HT_4$ | *E. coli* | 5–8 mg/l | 6 M urea<br>0.2% SDS | 6his Ct<br>KSI | $Ni^{2+}$ aff. chrom. | 0.3–0.5 mg/l | DMPC/CHAPS/CHS<br>1/1/0.02% | 13 |
| $h5HT_{1A}$ | *X. laevis* | 1–5 ng/tadpole<br>2.5 pmol/mg | 1 mM DM | EGFP Nt<br>$Rho_{15}$ tag | 1D4 immunoaff. | 3 ng/tadpole<br>0.16 pmol/mg | – | 108 |
| $hBLT_1$ | *E. coli* | 10 mg/l | 6M urea | 6his Ct | $Ni^{2+}$ aff. chrom. | 2–3 mg/l | LDAO | 12 |
| $ET_B$ | *P. pas* | 45pmol/mg | 1% DM | FLAG Nt<br>10his Ct<br>Bio tag Ct | 1: $Ni^{2+}$ aff. chrom.<br>2: FLAG immunoaff.<br>or monomeric avidin | 15 pmol/mg | – | 29 |

h, human; m, mouse; r, rat; p, porcine; b, bovine; t, turkey; *X. laevis, Xenopus laevis; E.coli, Escherichia coli; S.cer, Saccharomyces cerevisiae; P. pas, Pichia pastoris; Sf, Spodoptera frugiperda; Dm, Drosophila melanogaster; Tni, Trichoplusia ni;* COS, kidney african green monkey cell; HEK293, human embryonic kidney cell; CHO, Chinese hamster ovary cells; NS-20Y, neuroblastoma cells; mL1.2 pre-B cells, murine L1.2 pre-B cells; Cf2th, canine thymocytes Cf2th; L-cells, endocrine cells; $\alpha$, $\beta$, adreno receptor; NTS, neurotensin receptor; TSHR, thyroid-stimulating hormone receptor; $PTH_1$, parathyroid hormone receptor; LH/CGR, luteinizing hormone/chorionic gonadotropin receptor; FSHR, follicle-stimulating hormone receptor; D, dopamine receptor; $\mu$, $\delta$, opioid receptors; CB, cannabinoid receptor; OR, olfactive receptors; M, muscarinic acetylcholine receptor; Rho, rhodopsin; mGlu, metabotropic glutamate receptor; NK, neurokinin receptor; TP, thromboxane receptor; PAC*1*, pituitary adenyl cyclase-activating polypeptide receptor; $P2Y_{12}$, purinergic receptor; 5-HT receptor, hydroxyptamine receptor; BLT1, leukotriene B4 receptor; D6, chemokine D6 receptor; CCR, chemokine receptor; CXCR4, receptor for the chemokine stromal cell-derived factor (SDF-1$\alpha$); Ste, mating factor receptor; ET, endothelin receptor; LDAO, lauryl-di-methyl-amino oxide; CHAPS, 3-((3-cholamidopropyl) di-methy-lamino)-1-propanesulfonate; CHAPSO, 3-[(3-cholamidopropyl)di-methy-ammonio]-2-hydroxy-1-propanesulfonic acid; Na cholate, sodium cholate; DM, n-dodecyl-$\beta$-D-maltoside; DC, dodecanoyl sucrose; SML, sucrose monolaurate; CHS, cholesteryl hemisuccinate; OG, octyl glucoside; FC-12, dodecylphosphocholine; NLS, N-lauroyl sarcosine; NG, n-nonyl-$\beta$-D-glucoside; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; KT3, 8 amino acids epitope; his, serial histidine; FLAG, 8 amino acids epitope; Bio, 15–24 amino acids epitope; Strep, 8–9 amino acids epitope; Rho, 9 amino acids epitope from rhodopsin; $Rho_{15}$, DNA fragment encoding the last 15 amino acids of mouse rhodopsin; HA, 9 amino-acid epitope from hemagglutinin influenza virus; ND, not determined; c-myc, 10 amino-acid epitope from the myc oncogene; Ct, C-terminal position; Nt, N-terminal position; Aff. chrom, affinity chromatography; ABT, 3-(2´-aminobenzhydryloxy) tropane; GFP, green fluorescent protein; GST, glutathione-S-transferase; MBP, maltose-binding protein; TrxA, thioredoxin; NTR, neurotensin; Cat, cation; WGA, wheatgerm agglutinin; DEAE, di-ethyl-amino-ethyl; Seph, sepharose; KSI, ketosteroid isomerase; EGFP, enhanced green fluorescent protein; hFC, human IgG Fc fragment; DMPC, dimyristoylphosphatidylcholine; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PC, phosphatidyl choline; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPG, palmitoyl-oleoyl-phosphatidylglycerol; POPE, palmitoyl-oleoyl-phosphatidylethanolamine; DOPC, dioleoyl-phosphatidylcholine; LPC, lysophosphatidylcholine; DOP, 1,2-dioleoyl-sn-glycero-3-phosphate; Erg, ergosterol; PA, phosphatidic acid; DPPS, dipalmitoyl phosphatidylserine; DPPE, dipalmitoyl phosphatidylethanolamine; DMPG, dimyristoyl phosphatidylglycerol.

opioid receptor [30]. Mirzabekov et al. [33] used another method and tested a broad spectrum of detergent conditions to determine the detergent that allowed solubilization and isolation of native CCR5. The method was guided by the comparison between the amount of solubilized CCR5 capable of being precipitated by a conformation-dependent CCR5 antibody versus precipitation by an antibody directed against a linear non-conformational epitope tag.

**Solubilization of recombinant GPCRs**

Table 2 presents recombinant GPCRs for which both the solubilization and purification steps have been described. Heterologous expression has been realized in various systems such as prokaryotes (*E. coli*), yeasts (*P. pastoris, Saccharomyces cerevisiae*), insect cells (*Drosophila, Lepidoptera*) and mammalian cells (CHO, HEK, etc.). A rapid analysis of the data clearly reveals that the intrinsic nature of the receptor considered, the expression system employed (in particular the lipid composition of the corresponding membranes), the composition of the solubilization buffer and numerous other parameters (e.g. initial protein concentration, temperature, time allowed for solubilization to occur) can influence the efficiency of the solubilization of a particular membrane protein [18, 34, 35]. The solubilization studies performed on the CCR5 and CXCR4 chemokine receptors are prototypes: 18 different detergents were tested for solubilization of the CCR5 receptor [33] and a hundred different conditions were explored to extract the CXCR4 receptor [36]. These experiments show that among a family of recombinant GPCRs, expressed in a special organism, the solubilization agents and conditions differ from one receptor to another. Indeed, Cymal 5 (a non-ionic detergent), perfectly adapted to the solubilization of CCR5 in a functional form [33], is totally inefficient to extract CXCR4 in a functional form [36]. Moreover, the results also depend on the initial membrane environment. Hence, the functional solubilization of CXCR4 from canine thymocytes is feasible using 1% 3-[(3-cholamidopropyl) di-methyl-ammonio]-2-hydroxy-1-propanesulfonic acid (CHAPSO, a zwitterionic detergent) [36], while a mixture of DM and cholesterol hemisuccinate (CHS) is necessary to extract the endogenous receptor from human T lymphocytes [37]. Differences in the initial lipid composition of the membranes of these two cell types could explain this discrepancy.

Table 2 demonstrates that a low number of detergents like digitonin, 3-((3-cholamidopropyl)di-methyl-amino)-1-propanesulfonate (CHAPS, a zwitterionic detergent) and DM are of rather general use for GPCR solubilization and have been appropriate choices in many solubilization and purification experiments. In fact, these detergents often combine good efficiencies of solubilization with stability of proteins in the detergent-solubilized state [15]. Some GPCRs require the use of several detergents simultaneously for solubilization, and this considerably increases the complexity and the number of tests to perform in order to establish optimal solubilization conditions. For example, the combination of CHAPS, DM and CHS allowed solubilization of the neurotensin receptor with an efficiency of 85% whereas solubilization with CHAPS/CHS resulted in an efficiency of 35% against 30% with DM alone [38]. One should note that most of the solubilized GPCRs described in table 2 remained active in the lipid-protein-detergent mixed micelles formed, allowing investigators not only to follow the receptor using ligand-binding tests but also to consider purification strategies based on ligand-receptor affinities (see below on purification). Nevertheless, this kind of strategy does not give access to the total expression yield. Several studies show that the stabilization of the receptor using the tight binding of a specific ligand can increase the stability of the protein during solubilization and therefore favor its functional solubilization. For example, the functional solubilization of the human $\alpha_{2C}$ adrenergic receptor was only possible in the presence of an antagonist [39]. The stability of the rat $M_3$ acetylcholine (muscarinic) receptor solubilized in the presence of digitonin is also dependant on pre-stabilization of the receptor by a specific ligand [40]. However, the functional solubilization by a mixture of digitonin and CHS of human $M_1$, $M_2$, $M_5$ and rat $M_3$ and $M_4$ receptors is independent of pre-stabilization by any ligand [41]. The human $M_3$ receptor, common to these two studies, and expressed in each case in insect cells, seems to be stabilized in an active conformation in the presence of CHS. Some authors have shown that the initial functionality of recombinant GPCRs is not an absolute prerequisite for its functional reconstitution. This is the case for an olfactive receptor [9] and for $BLT_1$ and 5-$HT_4$ receptors [11–13] expressed in *E. coli*. In these examples, the recombinant receptors were expressed in inclusion bodies and were initially totally inactive in terms of ligand binding. Obviously, the optimization of the solubilization step was not conducted to preserve the protein activity, and the purified protein was subsequently refolded in a functional form into proteo-liposomes [10] or lauryl-di-methyl-amine-N-oxyde (LDAO) micelles [11–13]. However, maintaining a functional protein throughout the solubilization and subsequent purification procedure is advantageous because the reconstitution of an aggregated GPCR form is still a very challenging task.

**The purification step: principle and objectives**

Purification of membrane proteins such as GPCRs is not as easy as the purification of water-soluble proteins, although the methods used are generally the same. A typi-

cal membrane protein isolation protocol often involves, as a first step, the preparation of a receptor-enriched fraction prior to the use of detergents. In many cases, i.e. when the receptor is embedded in a membrane compartment, this initial step consists in the preparation of a crude membrane fraction after cell disruption. Alternatively, GPCRs can also be pre-purified from *E. coli* inclusion bodies by differential centrifugation. Whatever the original environment they are sitting in, GPCRs, which are hydrophobic molecules, tend to form aggregates even in the presence of detergents, a phenomenon which increases the difficulties encountered during their purification.

Chromatographic methods based on the intrinsic properties of the protein can be used. These methods rely on the chemical and physical properties of the protein (cationic, anionic, hydrophobic behavior) or on the presence of post-translational protein modifications such as glycosylation, which allow the use of lectin affinity chromatography. Gel filtration chromatography (also known as size exclusion or gel permeation chromatography), which separates molecules on the basis of their size, can also be used. The specificity of interaction of a recombinant receptor toward a chromatographic matrix can be modulated by the addition of specific tags thanks to molecular biology techniques. The most frequently used tag consists of several consecutive histidines (usually 6 to 10) at the N or C terminus of the protein. This tag presents affinity for a metal phase matrix. It is also possible to add an immunotag that binds to an antibody matrix, a glutathione S-transferase (GST) tag that binds to a glutathione phase or a Bio tag, which can be *in vivo* or *in vitro* biotinylated, and binds to a streptavidin matrix. These affinity tags do not allow discrimination between active and non-active receptor molecules. The purification of GPCRs can be performed on proteins that either have or have not retained their functional conformation during the solubilization procedure. According to the preservation of the binding properties, the adopted strategies may be different. For example, the active form of a receptor can be purified on a specific ligand column but also by immunoaffinity on a conformational antibody column.

## Chromatographic methods based on the intrinsic properties of the protein

These methods rely on the intrinsic properties of the receptors such as charge and molecular weight but also on the occurrence of post-translational modifications.

### Ion exchange chromatography

Depending on the isoelectric point of the receptor and the pH of the buffer, the protein can be chromatographed on cation or anion exchange columns [27, 42–45]. The solubilizing detergent must be chosen carefully depending on the ion type of the column phase, and in general, neutral detergents are preferable. Among the ion exchangers, one can mention heparin sepharose constituted of a highly sulfated glycosaminoglycan, which acts as a cation exchanger due to its high content of anionic sulfate groups [46] and hydroxy-apatite, which is an inorganic chromatographic material containing calcium phosphate, and thus containing both positive and negative charges. Even though the binding mechanisms are not completely understood, ionic interactions as well as adsorption effects seem to contribute to protein binding [43, 46].

### Gel filtration chromatography

Gel filtration chromatography, also known as size exclusion chromatography or gel permeation chromatography, separates molecules on the basis of their size. As shown in table 2, these chromatographic methods are often used as a complement to other methods [25, 38, 46–48]. This method is unable to give the molecular weight of the protein when it is solubilized in detergent, since the solubilized receptor is contained within detergent micelles. Thus, the mouse $\delta$-opioid receptor solubilized in DM and chromatographed on a gel filtration column migrated with an apparent molecular weight of approximately 340 kDa, whereas the molecular weight of the recombinant receptor is 46 kDa [47]. Tucker and Grisshammer [38] used an intermediate gel filtration chromatography in order to eliminate the biotin used to elute the biotinylated receptor from a monomeric avidin column.

### Lectin affinity chromatography

Lectin affinity chromatography was initially largely employed to determine the nature of the glycosylations added on the recombinant receptors expressed in a given expression system [40, 49]. This strategy is based on the affinity of glycans for immobilized lectins [50] and it obviously works on proteins expressed in organisms performing this specific type of post-translational modification, i.e. eukaryotic cells. Different categories of lectins are available and can be used to purify glycoproteins. Concanavalin A (ConA) or lentil lectin binds with a very high affinity to proteins presenting N-glycosylated amino acids with high (oligo)-mannosidic carbohydrates, but has a very low affinity for other glycoproteins. In contrast, wheat germ agglutinin (WGA) lectin selectively interacts with proteins containing complex N-glycans [50]. Proteins bound to lectin resins are specifically eluted with monosaccharides, $\alpha$-D-methyl mannoside or $\alpha$-D-methyl glucoside for ConA, and N-acetyl glucosamine for WGA. The human $\beta$2 adrenergic receptor expressed in Sf9 cells binds to a ConA resin, but does not interact with a WGA resin, and the authors concluded that the re-

ceptor mainly contains high (oligo)-mannosidic sugars [49]. In contrast, the rat $M_3$ receptor expressed in Sf9 cells was able to bind both on WGA and ConA resins, demonstrating a microheterogeneity in glycosylation [40]. This last result underlines one of the major limitations of lectin affinity chromatography for the purification of recombinant GPCRs: the glycan microheterogeneity of the protein to purify. Moreover, because of the presence of numerous glycosylated proteins capable of interacting with the resin employed, this type of chromatography must always be coupled to other purification methods, more specific for the target protein [38, 48, 51].

## Chromatographic methods based on the presence of specific tags on the protein

### Immobilized metal affinity chromatography

This chromatographic method uses the chelating and affinity properties of a set of serial histidines for divalent metal ions attached to an immobile phase. His-tag recombinant proteins are eluted from the column using a gradient of imidazole or by decreasing the pH of the buffer. The use of immobilized metal affinity chromatography (IMAC) has already been largely successful for the purification of soluble proteins either in denaturing or native conditions [52, 53]. This approach has subsequently been successfully employed for membrane protein purification in the presence of detergents [see for example refs. 54–57], using the divalent $Ni^{2+}$ cation most of the time. The results presented in Table 2 clearly show a very large utilization of the IMAC technique for GPCR purification. The efficiency of purification is dependent on a number of parameters such as detergent concentration or His-tag length and no consensus is found about the C terminus or N terminus position of the serial histidine tag. Investigation of the effect of His-tag length on purification has shown, for the neurotensin receptor expressed in *E. coli,* that the deca-histidine sequence was more efficient than the hexa-His tag [58]. In this work, a twofold increase in receptor enrichment was shown in one step, by exchanging a hexa-histine tag for a deca-histidine tag. The utilization of an extended histidine tail allowed stringent washes at high concentrations of imidazole in order to remove non-specifically bound contaminants. The importance of the detergent was also shown: in general, anionic detergents such as SDS or sarkosyl are not recommended, while non-ionic detergents such as DM or Triton X-100 can be employed up to 2%. Nevertheless, the hexa-His-tagged $\mu$-opioid receptor solubilized in 0.1% SDS could be purified by IMAC with an excellent yield [30]. The introduction of a C terminus deca-His tag to the $H_1$ histamine receptor allowed, after solubilization in DM, the purification, in a single step, of the recombinant protein with a purification yield higher than 90% and an excellent recovery of function (up to 70%) [28]. In addition, demonstrating once again that each receptor has a specific behavior, Pawate et al. [59] obtained only 14% purification yield and 36% recovery for the CHAPS-solubilized hexa-His-tagged thromboxane A2 receptor. After solubilization of a membrane fraction isolated by centrifugation on a sucrose gradient, the Ste2 receptor from *S. cerevisiae* was purified in a single step on a $Ni^{2+}$ resin [60], while the same strategy applied to the yeast over-expressed dopamine $D_1$ receptor did not lead to complete purification of the protein [61]. To purify the OR5 olfactive receptor, two serial Ni-NTA affinity columns were used [9]. This hexa-His-tagged receptor was expressed as a GST fusion protein and the first imidazole elution was able to produce a receptor-enriched fraction. After cleavage of the GST tag with thrombin, the sample was chromatographed again on an Ni-NTA column. Hence, combining different purification steps is sometimes necessary to increase the purification yield. Nevertheless, the coupling of this first affinity step on a nickel resin to a second one using the FLAG tag did not lead to the complete purification of the $D_1$ dopamine or $\beta_2$ adrenergic receptors [61, 62]. Finally, Hayashi and Haga [63] in 1996 showed that $Co^{2+}$ ions used instead of $Ni^{2+}$ cations can be used to purify to apparent homogeneity the human $M_2$ receptor. Lastly, the human neurokinin $NK_1$ receptor has been purified to homogeneity in two steps, a first IMAC step followed by gel filtration chromatography [25].

### Biotin-streptavidin affinity chromatography

This purification method uses the exceptional affinity of either avidin, a glycoprotein derived from egg-white, or its bacterial counterpart, streptavidin, for biotin (vitamin H). Bio-tag or Strep-tag can both be used as fusion tags. Bio-tag is a 15–24 amino acid sequence recognized by a biotin protein ligase, whose function is to covalently attach biotin to the Bio-tag. This tag can be biotinylated *in vivo* or *in vitro*. Tucker and Grisshammer [38] have shown that the $NTS_1$ neurotensin receptor with the Bio-tag fused to its C terminus can be purified to homogeneity in two steps. The crucial first step involved the purification of the *in vivo* biotinylated protein on a monomeric avidin column. This yielded essentially pure receptor with a remarkable 60-fold purification and 38% yield. The use of the Strep-tag, an 8–9 amino acid peptide sequence, which selectively binds streptavidin, was less efficient than that of the bio-tag [38].

Ohtaki et al. [51] used an original strategy to purify the $PAC_1$ receptor: the solubilized recombinant receptor was mixed with the biotinylated PACAP 38 ligand and purified on an avidin affinity gel. The fully active receptor was then further purified by lectin affinity chromatography.

**Large-tag affinity chromatography**

Large tags such as proteins can be used to help in the purification of GPCRs. For this purpose, the maltose-binding protein (MBP, 10 kDa) was fused to the $\beta_2$ adrenergic receptor [64]. The fusion protein was purified on an amylose column and the elution was performed using maltose in the buffer. The 27-kDa GST protein can also be used, and in this case, the fusion protein was purified on a glutathione column and the elution performed with glutathione [9].

In the experiments reported by Kiefer et al. [9], after solubilization in detergents, the OR5 olfactory receptor fused to GST was unable to bind to the glutathione matrix, probably because the GST domain was misfolded. More often, these protein tags have been used for their chaperone-like properties, assisting in correct protein folding and leading to active proteins [38].

**Ligand-based affinity chromatography**

This approach has been frequently employed to purify GPCRs from natural sources, particularly when recombinant protein technology could not be performed. It necessitates the production and solubilization of a fully active receptor able to bind a specific ligand chemically attached on a chromatographic phase. Purifying a GPCR this way is very advantageous since it both isolates the protein of interest from others and discriminates between active and non-active receptor molecules. The $\beta_2$ adrenergic receptor has been the most frequently purified receptor by means of ligand-based affinity chromatography using the specific ligand alprenolol [46, 49, 62, 64–66]. In the same way, acetylcholine (muscarinic) receptors have been purified on a 3-(2′-aminobenzhydryloxy) tropane (ABT)-agarose affinity chromatography gel [43, 46, 63]. ABT is an antagonist ligand that binds to muscarinic receptors with nanomolar affinity, and the elution of the receptor from the column is performed with atropine. The specific activity of the receptor was threefold higher when the receptor was purified with ABT sepharose compared with receptor purified with chelating sepharose. In their report, Zeng and Wess [67] showed that both monomeric and dimeric/oligomeric forms of a modified rat $M_3$ acetylcholine (muscarinic) receptor were capable of binding muscarinic ligands. In the case of the $A_{2a}$ adenosine receptor [42], an efficient ligand affinity chromatography using the antagonist xanthine amine congener was reported. The receptor was eluted using theophylline. However, a final ion exchange step was necessary to achieve the purification.

**Immunoaffinity-based chromatography**

This technique relies on the use of the very specific recognition between a monoclonal antibody and an epitope. The specific antibody can be directed either against the receptor [39, 68–74] or against a sequence fused to the recombinant receptor.

Several tags are often used as epitopes for immunoaffinity purification, such as the rhodopsin tag, which consists of the C-terminal 9 amino acids of bovine rhodopsin (also known as the rho tag), [33, 36, 75, 76], or the FLAG tag, which is the 8–9 amino acid leader peptide of the gene-10 product from bacteriophage T7 [26, 43, 48, 61, 62, 77, 78]. For this latter peptide, the affinity of the corresponding monoclonal antibody is $Ca^{2+}$ dependent, and elution of the bound receptor can either be performed with EDTA or the FLAG peptide. This FLAG tag is also cleavable with enterokinase [79].

As examples, the human $\beta_2$ adrenergic, CCR5, CXCR4 and $\alpha_{2C}$ adrenergic receptors [33, 36, 39, 51, 66, 80] have been purified by immunoaffinity chromatography. Except for the $\beta_2$ adrenergic receptor for which two steps were necessary [66], the three other GPCRs were purified to apparent homogeneity in a single step. In a few cases [36, 81], immunoprecipitation with protein A or protein G sepharose allowed preparation of enriched receptor fractions.

**Renaturation and structural biology of GPCRs**

Using a variety of detergents (Table 2), numerous GPCRs can be solubilized in membrane-mimetic environments with their binding activity preserved. Nevertheless, this detergent environment is quite different from their natural environment. Thus, the best environment for the functional reconstitution of GPCRs should be extracts from the original membrane they are sitting in. In the context of structural biology experiments, a complex environment is not desirable and reproducibility is not guaranteed with biological membranes. This explains why protein reconstitution is very often performed in artificial membranes (Table 2). In the same way as different detergents must be tried for solubilizing a given receptor [33, 36], different model membrane compositions must be tested for receptor reconstitution. For example a *Drosophila melanogaster* metabotropic receptor was solubilized in the detergent lyso-phosphocholine-12. After exchanging it for $\beta$-octyl-glucoside, the receptor was reconstituted into various concentrations of phosphatidylethanolamine, phosphatidyl-choline, phosphatidic acid and cholesterol. The receptor was found inserted into liposomes in all cases, independently of the lipid composition, but recovery of glutamate binding was strictly dependent on the presence of ergosterol (the sterol originally found in *Drosophila* membranes). Then, the optimal concentration of ergosterol was determined, and the highest specific binding was obtained with 15% ergosterol. In the same way, cholesterol has been reported to modulate

the functional activity of other GPCRs [82–84]. Efficient restoration of ligand-binding activity upon reconstitution of a GPCR in artificial membranes may also require the addition of catalytic amounts of natural membranes. This is the case for the *S. cerevisiae* $\alpha$ factor receptor [60]. After solubilization in 2% DM, the receptor was purified on a Ni-NTA column and reconstituted in artificial vesicles composed of 60:40 1-palmitoyl-oleyl-phosphatidylcholine:1-palmitoyl-oleylphosphatidyl-glycerol. The specific activity of the reconstituted receptor indicated that only 6% of the receptor was capable of ligand binding, but the addition of solubilized plasma membranes from *S. cerevisiae* to the artificial membranes restored most of the expected ligand activity (at least 80%). Nevertheless, the co-factors responsible for this effect were not identified [60].

Once solubilized, full-size GPCRs can be directly structurally characterized in a soluble form. One of the easiest methods employed to characterize receptor refolding is circular dichroism (CD). This spectroscopic method allows the determination of the secondary-structure content of the receptor and was used for the porcine $M_2$ acetylcholine (muscarinic) receptor solubilized in 0.1% digitonin, 0.02% cholate [85], for the mouse 5-HT4 receptor solubilized in dimyristoylphosphatidylcholine/3-((3-cholamidopropyl) di-methyl-amino)-1-propanesulfonate/cholesterol (1%/1%/0.02%) [13] and for the human leukotriene B4 receptor solubilized in 2 mM LDAO [12]. As shown in all these studies, the far-UV spectra in the 200- to 250-nm range were characteristic of a folded protein with a high content of secondary structures, ~50% of these being $\alpha$-helical. These results were consistent with the generally admitted seven transmembrane-spanning structure for the GPCRs. In addition, the analysis of the CD spectra in the near-UV region (250–310 nm) of the 5-$HT_4$ and $BLT_1$ receptors demonstrated the occurrence of a tertiary fold made of a disulfide bridge between extracellular loops. Despite these interesting results, no detailed 3D structural information is available yet for full-size recombinant GPCRs. Nevertheless, two very promising pathways in the structural biology of GPCRs are currently being developed. The first consists in the determination of the receptor-bound conformation of specific ligands. This was performed using NMR techniques for two acetylcholine analogues bound to the $M_2$ acetylcholine (muscarinic) receptor [7], for the pituary-adenylate-cyclase-activating polypeptide bound to the $PAC_1$ receptor [5], for the neurotensin peptide bound to the $NTS_1$ neurotensin receptor [8] and by X-ray crystallography for the glutamate bound to the extracellular domain of the $mGlu_1$ receptor [6]. All these results were enabled by the availability of milligram quantities of pure receptor. The second research field concerns the structure of extracellular domains of GPCRs [for a classification of GPCRs see ref 86]. These domains present ligand-binding properties, are located in the N-terminal part of the receptor and hence show the same characteristics as water-soluble proteins. The conformation of the soluble ligand-binding domain of the glutamate receptor [6], the ectodomain of Methuselah, a *Drosophila* GPCR [87], the extracellular cysteine-rich domains at the amino terminus of Frizzled proteins [45] and the extracellular domain of human follicle-stimulating hormone receptor in complex with its hormone [88] were determined by X-ray crystallography, and the conformation of the extracellular domain of the corticotropin-releasing factor by NMR [89].

## Conclusion

The difficulties to get, at atomic resolution, structural information on GPCRs arise largely from the problems encountered during their solubilization and purification. A great number of research consortiums have been created in recent years to set up high-throughput screening strategies for the determination of 3D structures of GPCRs [90, 91]. The various steps involved in this strategy include vector production, receptor expression, expression scaling up, solubilization, purification, refolding and structural biology. At present, different efficient recombinant GPCR expression experiments have been reported [4], and the solubilization and purification steps are well advanced. Together with other research teams all over the world that do not engage in high-throughput screening strategies but are concentrated on a particular GPCR, we are confident that in the near future, an increasingly number of GPCR structures will be obtained at atomic resolution.


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