

New advances in production and functional folding of G-protein-coupled receptors

Jean-Louis Banères^{1,2}, Jean-Luc Popot^{3,4} and Bernard Mouillac^{5,6,2}

¹ CNRS, UMR-5247, Institut des Biomolécules Max Mousseron, Faculté de Pharmacie, 15 avenue Charles Flahault, F-34000 Montpellier, France

² Universités de Montpellier 1 & 2, F-34000 Montpellier, France

³ CNRS, UMR-7099, Laboratoire de Physico-Chimie Moléculaire des Protéines Membranaires, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75000 Paris, France

⁴ Université Paris-7, F-75000 Paris, France

⁵ CNRS, UMR-5203, Institut de Génomique Fonctionnelle, Département de Pharmacologie Moléculaire, 141 rue de la Cardonille, F-34000 Montpellier, France

⁶ INSERM, U661, F-34000 Montpellier, France

G-protein-coupled receptors (GPCRs), the largest family of integral membrane proteins, participate in the regulation of many physiological functions and are the targets of approximately 30% of currently marketed drugs. However, knowledge of the structural and molecular bases of GPCR functions remains limited owing to difficulties related to their overexpression, purification and stabilization. The development of new strategies aimed at obtaining large amounts of functional GPCRs is therefore crucial. Here, we review the most recent advances in the production and functional folding of GPCRs from *Escherichia coli* inclusion bodies. Major breakthroughs open exciting perspectives for structural and dynamic investigations of GPCRs. In particular, combining targeting to bacterial inclusion bodies with amphipol-assisted folding is emerging as a highly powerful strategy.

Overexpression of membrane proteins for structural and biophysical studies: still a challenge

Structural information on integral membrane proteins (IMPs) remains limited. According to databases of known 3D structures of IMPs (http://blanco.biomol.uci.edu/Membrane_proteins_xtal.html; <http://www.mpdb.tcd.ie>; <http://www.drorlist.com/nmr/MPNMR.html>), the crystal or NMR structure of only ~280 IMPs has been solved to date, compared to tens of thousands for soluble proteins. Worse, only a small fraction (~35%) of these proteins are eukaryotic, even though IMPs constitute 20–30% of the proteins encoded by human and other eukaryotic genomes. Among IMPs, G-protein-coupled receptors (GPCRs), whose transmembrane region is a seven-helix bundle, constitute the largest family [1]. More than 800 GPCRs have been identified, representing 2–3% of the coding sequences in the human genome. They are involved in most essential cellular processes and are the targets of approximately 30% of current pharmaceutical drugs. It is therefore critical to gain detailed knowledge of their structures and their

dynamics to understand their functions and/or dysfunctions and for the rational design of selective therapeutic compounds. However, except for rhodopsin, whose crystal structure was solved following its extraction from the retina [2,3], the low natural abundance of GPCRs generally precludes their purification in biochemically relevant amounts. Overexpression is thus a prerequisite to investigating GPCR structure or analyzing conformational transitions on interaction with ligands or with signaling proteins such as G proteins and arrestins.

Unfortunately, overexpression of GPCRs is still problematic and often results in low yield and protein aggregation or misfolding, if not cell toxicity. Consequently, although crystal structures of ligand-bound β_1 - and β_2 -adrenoceptors [4,5] and adenosine A_{2A} [6], chemokine CXCR4 [7] and dopamine D3 [8] receptors have been reported, investigation of the structure and dynamics of most GPCRs remains a daunting task. Many overexpression systems have been tested. GPCRs have been successfully produced by cell-free synthesis [9] and by heterologous expression in mammalian [10] and insect [11] cells, in photoreceptor cells of *Drosophila* [12], *Xenopus* [13] and mouse [14], and in microbes such as yeasts and bacteria [15,16]. As discussed below, expression in *Escherichia coli* holds great promise, not only because of its simplicity, rapidity, safety, scalability and genetic tractability, but also in terms of the quantity and homogeneity of the recombinant protein.

Once efficient expression has been achieved, purification of sufficient amounts of native-like, functional and stable protein remains a formidable challenge. GPCRs, as all IMPs, have to be handled in aqueous solutions in complex with surfactants, usually detergents. Because detergents tend to be inactivating, identification of a detergent or lipid-detergent mixture that ensures protein homogeneity, functionality and stability is often a limiting step. Nevertheless, several overexpressed GPCRs have been successfully purified in a stable (often engineered) and functional form, allowing their crystallization and

Corresponding author: Mouillac, B. (bernard.mouillac@igf.cnrs.fr).

structure determination. For instance, the adenosine A_{2A} receptor has been purified in a fully functional form and crystallized in *n*-dodecyl- β -D-maltopyranoside (DDM) mixed with cholesterol hemisuccinate (CHS) [6]. The β_1 -adrenoceptor has been crystallized in octylthioglucoside [4], whereas the β_2 -adrenoceptor was best solubilized, purified and crystallized in DDM [5]. DDM was also compatible with NMR spectroscopic analysis of the β_2 -adrenoceptor [17]. Although DDM is often used, it seems that an optimized surfactant environment has to be identified for each GPCR. Amphipols (APols), a new class of polymeric surfactants, are mild substitutes for detergents and constitute a very promising stabilizing medium for IMPs [18]. This review focuses on GPCR bacterial expression and on their functional folding using APols.

GPCR overexpression in bacteria: targeting the inner membrane or inclusion bodies?

GPCRs have been overexpressed in bacteria using two different approaches. GPCRs are plasma membrane proteins, so targeting of recombinant receptors to the inner membrane of the bacterium was initially considered as the most obvious strategy (Figure 1). In most cases, however, this leads to severe cell toxicity and low levels of expression. A more efficient insertion into the bacterial inner membrane can be achieved by fusing the GPCR to a protein helper partner. Thus, coupling of *E. coli* β -galactosidase

(114 kDa) to the N terminus of the human β_2 -adrenoceptor led to measurable membrane expression [19]. The combination of *E. coli* maltose-binding protein (MBP, 43 kDa), used as an N-terminal fusion partner, with *E. coli* thioredoxin A (TRX, 10 kDa), added at the C terminus of the GPCR, is particularly well adapted for expression of the rat neurotensin NTS1 and cannabinoid CB2 receptors [20]. The human adenosine A_{2A} receptor has been highly expressed with only MBP fused at the N terminus [21]. Fusion of the jellyfish GFP (27 kDa) to the C terminus of the human cannabinoid CB1 and bradykinin B₂ receptors has led to efficient membrane expression [22]. Although membrane expression of the NTS1 receptor was highly successful and has been applied to automated large-scale purification [23], the MBP-GPCR-TRX fusion strategy cannot be generally applied without extensive receptor truncations or modifications.

Expression of heterologous proteins in *E. coli* is frequently associated with incorrect folding and accumulation of the recombinant protein in cytoplasmic aggregates named inclusion bodies (IBs). Targeting of GPCRs to IBs combines many advantages. IBs are mechanically stable and can be easily isolated from other cell constituents by centrifugation, they are not toxic to the cell, and they are resistant to proteolytic degradation. GPCR production in IBs can be massive (Table 1). This implies, however, that the receptors thus expressed have to be subsequently

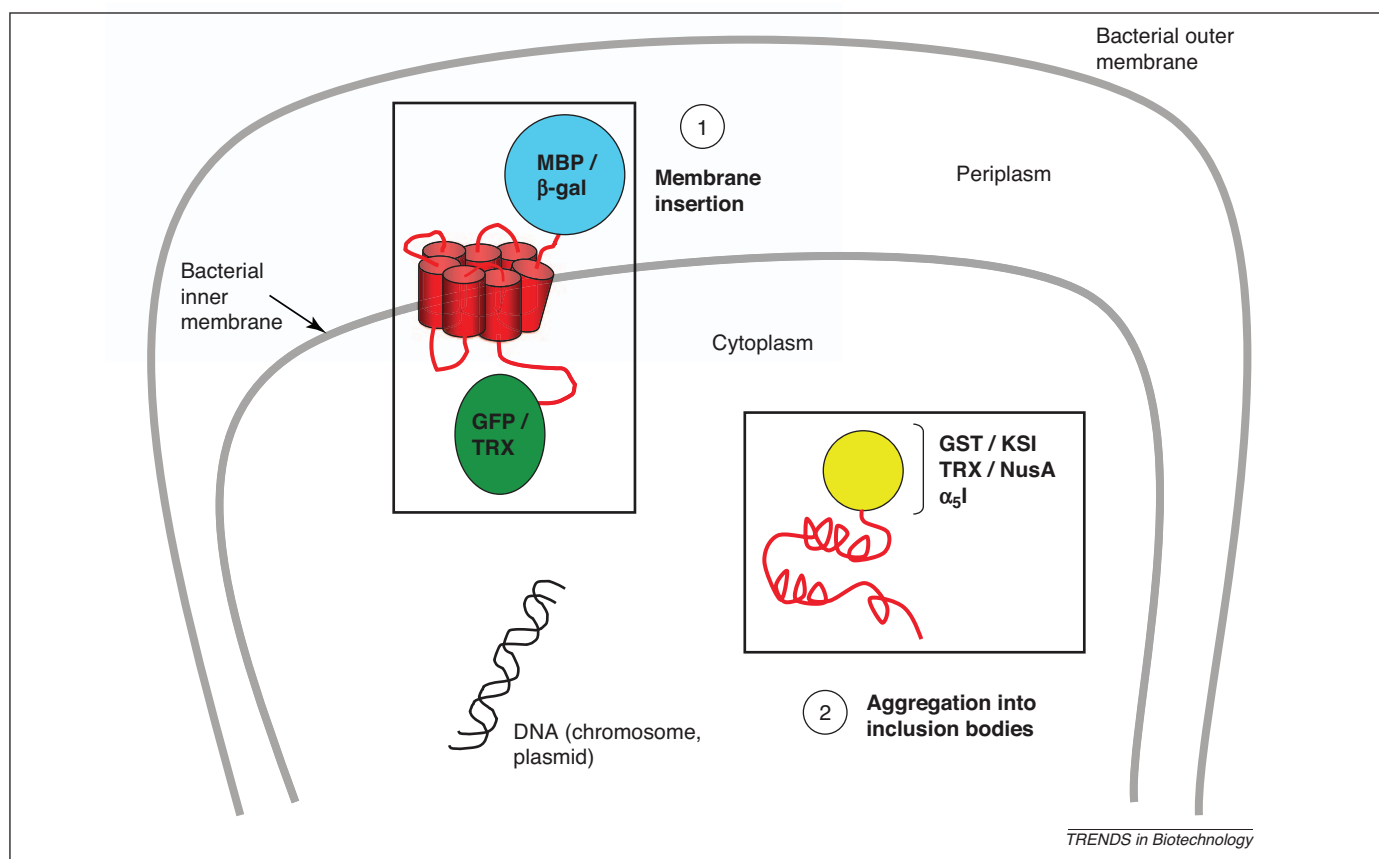


Figure 1. Strategies for overexpression of GPCRs in *E. coli*. GPCRs can be produced in bacteria either by (1) insertion into the inner membrane or (2) accumulation in inclusion bodies (IBs). In the first case, a protein partner is coupled at the N terminus (MBP, β -gal) or C terminus (GFP, TRX) of the receptor, or at both extremities (e.g. MBP and TRX) to target the recombinant protein to the membrane. In the second case, targeting to IBs is favored by coupling to the N terminus of another fusion partner, such as GST, KSI, TRX, NusA or α_5 . GPCRs accumulated as IBs are not adequately folded and have to be solubilized in a harsh detergent before folding.

Table 1. Level of production of several purified GPCRs following expression in *E. coli*

GPCR ^a	Fusion partner(s)	Site of expression	Yield (mg/L) ^b	Refs
Neurotensin NTS1	MBP+TRX	Inner membrane	0.13 ^c	[20]
Adenosine A2a	MBP	Inner membrane	0.17	[21]
Olfactory OR5	GST	IBs	0.2	[24]
Leukotriene BLT1	T7 tag	IBs	2–3	[25]
Serotonin 5HT _{4A}	KSI	IBs	0.5	[26]
Cannabinoid CB1	None	IBs	100 ^c	[28]
Vasopressin V2, leukotriene BLT2	α_5 I	IBs	0.8–1.2	[29]

^aFor each GPCR, the fusion partner(s) and site of expression in the bacterium, namely the inner membrane or inclusion bodies (IBs), are indicated.

^bIn each case, the quantity of pure and functional GPCR that has been obtained is indicated in mg/L of cell suspension. A functional receptor means that it has been overexpressed, separated from its fusion partner (if applicable) by enzymatic cleavage, folded and/or purified and its activity checked by ligand binding.

^cExcept for CB1 and NTS1, which were produced in fermenters, all other receptors have been overexpressed in culture flasks.

folded to their native state, which constitutes a difficult challenge (see below). This strategy was first successfully developed for the rat olfactory OR5 receptor and several other GPCRs [24], and subsequently improved for the human leukotriene BLT1 and the human serotonin 5-HT_{4A} receptors [25,26]. In most cases, a fusion partner is again needed for efficient production (Figure 1). Except for the BLT1 receptor, which has been expressed in IBs after fusion to a short T7 tag [25], most GPCRs were coupled to a large fusion partner such as the schistosomal glutathione S-transferase (GST, 25 kDa), and had to be truncated at their N terminus. The serotonin 5HT_{4A} receptor was efficiently expressed after fusion to bacterial ketosteroid isomerase (KSI, 12 kDa) [26], but KSI proved to be inefficient for other receptors (J-L. Banères, unpublished data). A recent high-throughput effort at large-scale production of more than 100 GPCRs as bacterial IBs revealed that a majority of them can be expressed in quantities sufficient for solubilization and purification [27,28]. This extensive study evaluated the efficiency of various fusion partners, namely GST, MBP, TRX and the *E. coli* N-utilization substance A (NusA, 50 kDa), to target GPCRs to IBs. Depending on the culture conditions, GST and TRX were identified as most efficient, although some GPCRs could be overexpressed without any protein tag.

Use of an α_5 integrin fragment (α_5 I, 31 kDa) as a targeting partner has facilitated the expression of many rhodopsin-like GPCRs at high levels, regardless of their length [337–472 amino acids (aa)], the selectivity of their G protein coupling or the nature of their endogenous ligands. This efficient and apparently generic procedure has been successfully applied to expression of the β_3 -adrenoceptor, the vasopressin V2 and V1b and oxytocin OTR receptors, the chemokine CCR5 and CXCR4 and chemokine-like ChemR23 receptors, the ghrelin GHS-1a receptor, the cannabinoid CB1 receptor and the leukotriene BLT1, BLT2, CysLT1 and CysLT2 receptors, without requiring any optimization of the GPCR coding sequence, the cell culture conditions or the extraction and purification procedures [29,30]. The α_5 I fusion strategy represents an important breakthrough for *in vitro* studies aimed at understanding the molecular basis of GPCR function and structure, and potentially for other membrane protein families.

Comparison of the different expression strategies in *E. coli* (Table 1) suggests that GPCR targeting to IBs has

interesting potential in terms of both the amounts produced and general applicability.

Functional folding of GPCRs from IBs using classical surfactants: the state of the art

Following expression, IBs are first solubilized under denaturing conditions. Then, following purification (using a metal-affinity chromatography procedure for instance), the fusion partner used for high-level expression has to be removed, usually through proteolytic cleavage. A notable advantage of α_5 I-GPCR fusions regarding this crucial biochemical step is that α_5 I keeps the receptor soluble after dialysis in the absence of denaturing agents, which greatly facilitates efficient proteolytic cleavage of the fusion protein [29]. After another purification step in sodium dodecyl sulfate (SDS) buffers, folding is then initiated by transfer from SDS to other surfactants (Figure 2). The folding efficiency depends on the competition between protein aggregation and 3D structure formation, as well as on the ability of the receiving surfactant to stabilize the native 3D state of the folded receptor [31].

Efficient folding implies that the solubilized protein is not aggregated to start with. Globular proteins expressed in IBs can be efficiently solubilized by chaotropic agents such as urea and guanidinium chloride. By contrast, IMP solubilization requires harsh detergents such as SDS or *N*-lauroylsarcosine (or organic solvents). In SDS, proteins in general, and in particular IMPs such as bacteriorhodopsin (BR) [32], the μ -opioid receptor [33] and the small multi-drug transporter EmrE [34], retain or acquire a significant amount of α -helical secondary structure. The α -helical content of the μ -opioid receptor determined in 0.1% SDS solution is ~40% at pH 7–8 [33], which is slightly less than for the predicted secondary structure of the full-length protein (50–54%). Given that some of the helical segments present in SDS solution are likely to overlap regions that form transmembrane helices in the folded protein, an SDS-solubilized GPCR should probably be considered not as fully unfolded, but rather as partially prefolded, at least as far as the secondary structure is considered.

GPCR folding is initiated by displacing the denaturing detergent with a milder surfactant. Under these conditions, regions that have a propensity to fold can do so, which leads to native-like interactions between folded segments. These can be intramolecular, which can lead to correct folding, or intermolecular, which can lead to

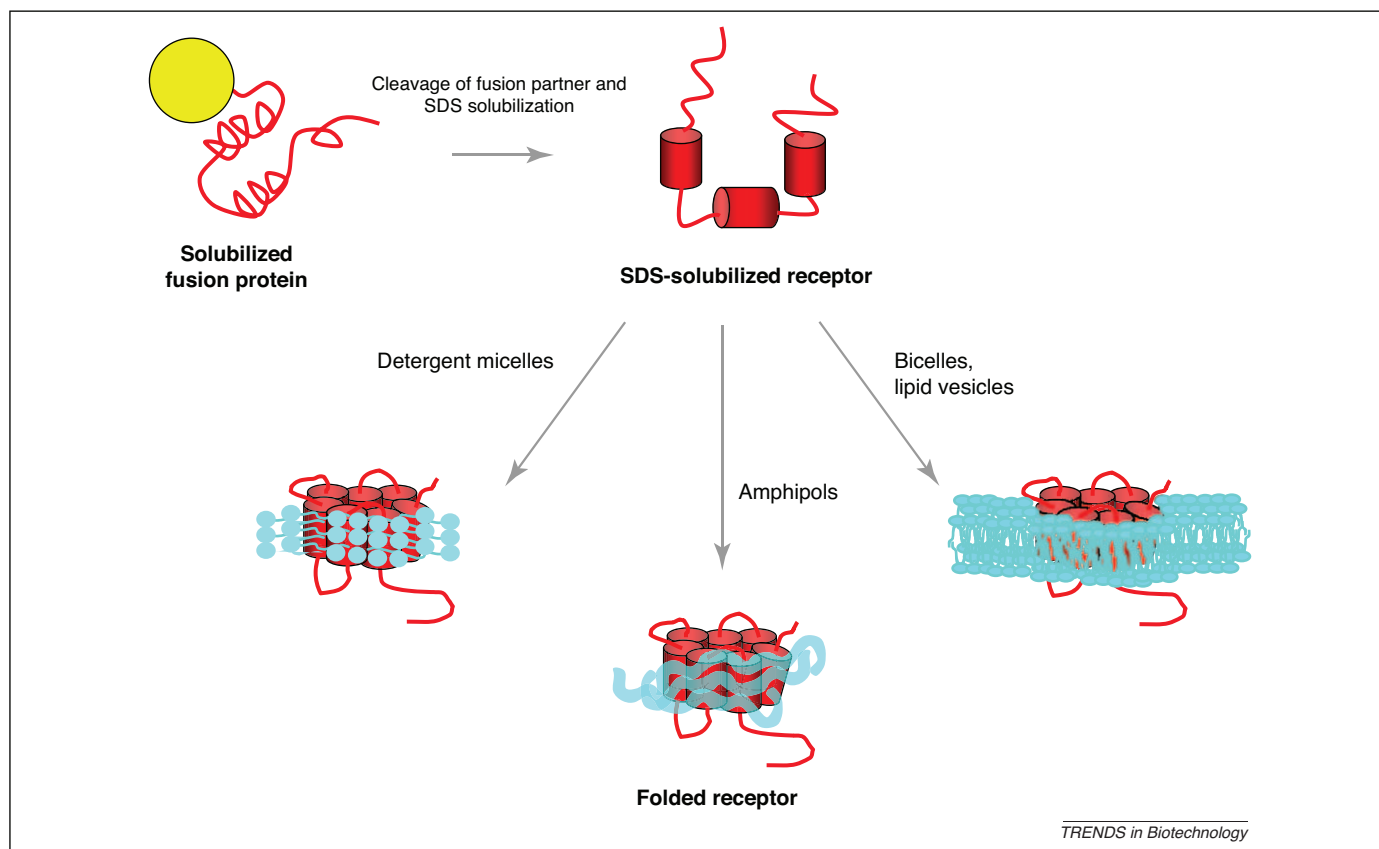


Figure 2. Strategies for folding GPCRs from IBs. Before folding, the recombinant GPCR accumulated in IBs has to be solubilized in denaturing buffers (e.g. a mix of urea and SDS), purified by immobilized-metal affinity chromatography (IMAC) and dialyzed in an aqueous buffer to eliminate most SDS and facilitate removal of the fusion partner, for instance via thrombin cleavage. After elimination of the cleaved fusion partner through a second IMAC purification under denaturing conditions, the GPCR is kept soluble in SDS solution, in which it displays a significant α -helix content. Folding can be achieved by transfer from SDS to mild detergents or amphipols, with or without lipid supplementation. The functional fraction of the GPCR preparation can be evaluated using pharmacological assays (ligand binding, activation of purified G proteins or arrestins).

aggregation. Finding favorable folding conditions therefore implies identifying a surfactant or surfactant mixture that will favor intramolecular interactions and then efficiently stabilize the native fold of the protein. Various such environments have been reported so far, although the limited number of successful examples makes it difficult to infer general rules. Refolding environments include classical detergents and lipid-detergent mixtures, bicelles, lipid vesicles and original surfactants such as APols (Figure 2).

Efficient folding in detergents has been reported for a limited number of GPCRs such as the leukotriene receptors BLT1 and BLT2 (Table 2). BLT1 was folded as a functional protein to $\sim 30\%$ in lauryldimethylamine oxide (LDAO) [25] whereas the BLT2 receptor was folded to its functional state to $\sim 4\%$ in *n*-dodecyl phosphocholine (DPC)-hexadecyl- β -D-maltoside (HDM) mixtures [29]. In both cases, addition of lipids (e.g. asolectin) was required to improve the percentage of functional recovery. It has been reported that other GPCRs efficiently fold in detergent micelles. As stated above, the OR5 receptor was first folded in digitonin before insertion in lipid vesicles [24]. Fluorescence-monitored ligand binding assays demonstrated that approximately 80% of the folded OR5 receptor bound its lilyal ligand [24]. More recently, refolding of the SDS-solubilized parathyroid hormone receptor 1 (PTH-1R) and the CB1 receptor was performed by exchanging SDS for a mixture of the non-ionic detergents DDM and Cymal 6

[28]. In this study, ligand binding assays demonstrated that $\sim 30\%$ of the folded CB1 was functional. It has been reported that the glucagon-like peptide-1 receptor (GLP-1R) folds as a functional protein to $\sim 40\%$ upon transfer from SDS to Brij78 [35]. In all these cases (PTH-1R, CB1 and GLP-1R) the folding process was carried out in the presence of methyl- β -cyclodextrin to strip off SDS. Removal of dodecylsulfate can also be achieved by precipitation using K^+ ions (see below) [36].

The efficiency of alternative membrane-like environments to fold and stabilize GPCRs recovered from IBs has also been explored. Certain mixtures of long- and short-chain phospholipids assemble as bilayer discs, called bicelles, that mimic the membrane environment (Figure 2). A limited number of GPCRs, specifically the serotonin 5-HT_{4A} [26] and neuropeptide Y₂ receptors [37], have been folded to a native-like conformation in dimyristoyl phosphatidylcholine (DMPC)-CHAPS bicelles with folding yields of $\sim 25\%$ and $\sim 65\%$, respectively.

A few cases of successful folding in lipid vesicles for GPCRs recovered from IBs have also been reported. The first such example was described in pioneering work by Kiefer's group on the OR5 olfactory receptor, in which the overexpressed receptor was solubilized in the strong, negatively charged detergent *N*-lauroylsarcosine and then folded by transfer to the non-denaturing detergent digitonin [24]. The digitonin-folded receptor was able to bind its

Table 2. Comparison of GPCR folding yields obtained in different surfactants

GPCR	Folding medium	Average maximum folding yield (%) ^a	Refs
BLT1	Detergent–lipid mixed micelles (LDAO–asolectin)	30	[25]
	A8-35	50	[44]
	A8-35–asolectin	65	[44]
BLT2	Detergent–lipid mixed micelles (DPC–HDM–asolectin)	4	[29]
	A8-35	50	[44]
	A8-35–asolectin	70	[44]
5-HT _{4A}	DMPC–CHAPS bicelles	25	[26]
	A8-35	30	[44]
	A8-35–asolectin	60	[44]
CB1	Detergent–lipid mixed micelles (Fos–choline–16–asolectin) ^b	0	[44]
	A8-35	30	[44]
	A8-35–asolectin	40	[44]
	Detergent mixed micelles (DDM–Cymal 6)	30	[28]

^aThe folding yield is the amount of functional protein obtained after folding (based on binding of specific ligands) compared to that of protein in SDS solution (based on A₂₈₀ measurements).

^bNo extensive detergent screening was carried out to optimize folding of the CB1 receptor in detergent–lipid mixed micelles.

ligand (see above), thus providing yet another example of successful folding in detergent. The OR5 receptor was subsequently reconstituted in lipid vesicles by supplementing it with DDM–palmitoyloleoyl glycerophosphocholine (POPC)–palmitoyloleoyl glycerophosphoglycerol (POPG) mixtures before removing the detergent by adsorption onto hydrophobic beads. Under such conditions, the protein was stabilized in a fully ligand-competent state (~1% of the solubilized and purified material), as assessed by photoaffinity labeling.

As in the case of bicelles, successful folding of GPCRs by direct transfer to lipids is limited to a few examples, such as the human neuropeptide Y₁ receptor [38].

Amphipol-assisted folding of GPCRs: a generic approach?

As summarized in the previous section, folding in detergents or detergent–lipid mixtures has thus far yielded only a handful of functional GPCRs. Moreover, even for the GPCRs that have been folded under such conditions, folding yields are usually low, conditions are highly idiosyncratic and their identification is very time-consuming. Development of a more general approach to folding GPCRs recovered from IBs would be of great interest. This has led to testing APols as a possible generic folding medium.

APols were initially designed, synthesized and validated as mild alternatives to classical detergents [39]. They are defined as ‘amphipathic polymers that are able to keep individual IMPs soluble under the form of small complexes’ [18,40–42]. What is of interest here is that: (i) most IMPs are more (and generally much more) stable in APols than they are in detergent solutions [18,40]; and (ii) it has been proven that APols are an efficient medium in which to fold IMPs to their native state [43,44]. APols are relatively short polymers (mass is typically 8–20 kDa) that carry a high density of both hydrophobic chains and highly hydrophilic groups. The prototypal APol, named A8-35 (Figure 3a) [39], remains by far the most thoroughly studied and most widely used APol [18,45,46]. The high solubility of A8-35 in water is due to the presence of carboxylate groups. As a consequence, A8-35 becomes insoluble in

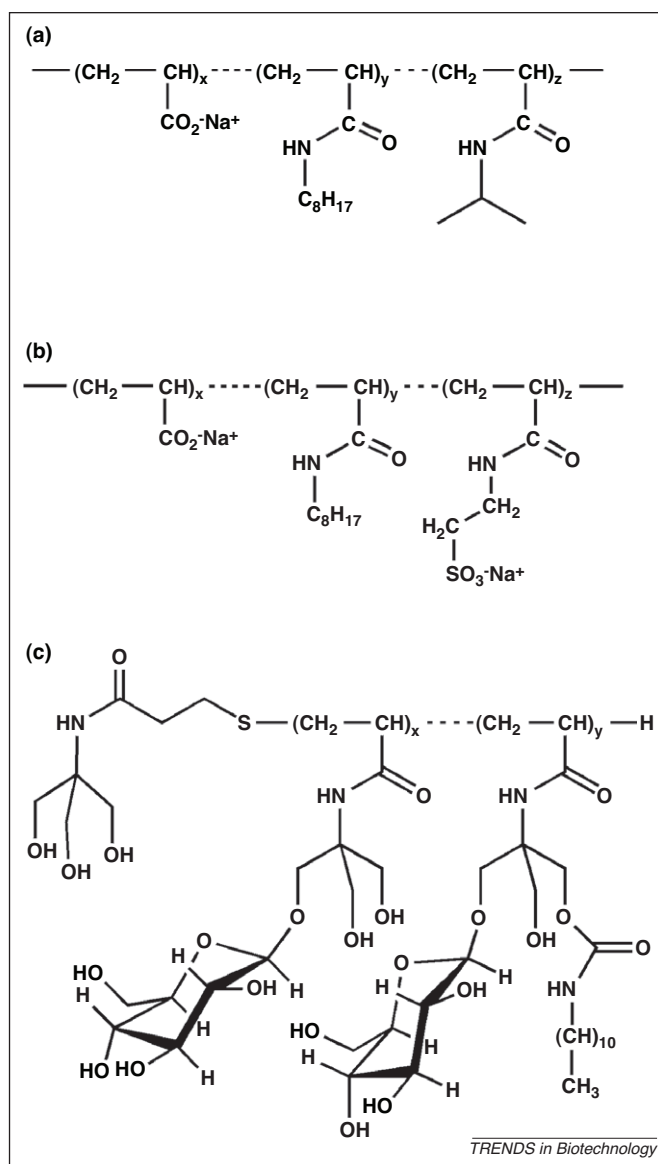


Figure 3. Chemical structure of three families of APols: (a) A8-35, the prototypal APol; (b) sulfonated APol (SAPol); and (c) glucose-based non-ionic APol (NAPol).

acidic solutions [45,46], a limitation that has prompted the development of alternative chemical structures such as sulfonated APols (SAPols; Figure 3b) [47] and glucose-based non-ionic APols (NAPols; Figure 3c) [48,49], both of which are insensitive to pH. In aqueous solution, APols form small micelle-like particles, each of which comprises only a few APol molecules (approx. four in the case of A8-35) [46].

IMP-APol complexes are typically obtained by one of the two following routes. Usually, a native IMP in detergent solution is supplemented with APols. This results in the formation of ternary complexes [50,51]. The detergent is then removed, yielding small compact IMP-APol complexes [52] in which the APol forms a thin layer covering the hydrophobic transmembrane surface of the protein [53,54]. Alternatively, a denatured IMP in SDS and/or urea is transferred to APols, during which process it recovers or adopts its native 3D structure (Figure 2 and see below). As a rule, APol-trapped IMPs are much more stable than their detergent-solubilized counterparts [18,40]. The underlying mechanisms are several. An essential factor is that APols are less efficient than detergents at disrupting the protein-protein and protein-lipid interactions that determine and stabilize the 3D structure of IMPs [40,47]. This led to the suggestion that in addition to being less aggressive towards properly folded, native IMPs, APols might provide a favorable medium for the formation or reformation of native-like interactions starting from a denatured protein.

APol-mediated IMP folding was first demonstrated using as models urea-solubilized OmpA and FomA, two β -barrel outer membrane proteins (OMPs) from the eubacteria *E. coli* and *Fusobacterium nucleatum*, respectively, and a paradigmatic α -helical IMP, BR, a light-driven proton pump from the plasma membrane of the archaeobacterium *Halobacterium salinarum* [43]. In the latter case, the SDS-solubilized BR was folded by precipitating dodecylsulfate as its potassium salt [36] in the presence of A8-35. The rationale behind the choice of this particular procedure is to proceed as rapidly as possible to the exchange of SDS for APols, leaving the protein little chance to explore misfolding or aggregation opportunities offered by partially denaturing environments. Precipitation achieves this goal much more efficiently than dialysis, adsorption of the detergent onto BioBeads or cyclodextrins, or exchange of surfactants after immobilization of the protein onto an affinity chromatography column.

It is truly remarkable that APols favor the folding of two IMP families with such completely different structures as OMPs and BR, which suggests that the approach could be quite general. This led to testing it on GPCRs recovered under denaturing conditions (in the presence of SDS) from *E. coli* IBs [44]. Conditions initially established for BR refolding were applied essentially without any changes to the folding of six GPCRs, namely the leukotriene B₄ receptors BLT1 and BLT2, the serotonin receptor 5-HT_{4A}, the cannabinoid CB1 receptor [44] and, more recently, the ghrelin GHSR-1a and the vasopressin V2 receptors (J-L. Banères and B. Mouillac, unpublished data). Folding yields of 30–50% were systematically achieved, depending on the receptor considered (these determinations were

based on ligand binding studies). This increased to 60–70% in the presence of lipids (Table 2). The presence of lipids increases not only the folding yield, but also the stability of APol-trapped GPCRs [44]. One possibility is that they do so by binding to sites that form when the transmembrane surface achieves its native state. Thus, they would contribute to steering folding towards the latter. As observed for most APol-trapped MPs, GPCRs folded in A8-35 are significantly more stable than those kept in lipid-detergent mixtures [44], which is of great interest for subsequent investigations [55]. The BLT1 and GHSR-1a receptors have also been folded in NAPols, with yields similar to those achieved in A8-35 (J-L. Banères, unpublished data). Although less thoroughly studied than A8-35, NAPols can be of interest when the purified proteins under study must be handled or studied at acidic pH, when their ligands tend to interact with polyanions such as A8-35, as is the case for ghrelin and vasopressin, or when studying the kinetics of interaction of G proteins with activated GPCRs, which is slowed down in the presence of A8-35 (J-L. Banères, unpublished data).

From a fundamental point of view, we note that seven α -helical IMPs (BR and six GPCRs) have now been successfully folded using a synthetic polymer, APol A8-35, whose chemical structure and supramolecular organization bear no similarity, beyond the amphiphilic character, to lipid bilayers. This indicates that, at least for these proteins, neither an environment mimicking the highly complex and anisotropic lipid bilayer nor even the presence of lipids is required for transmembrane helices to form and correctly orient and pack with each other, and for the polypeptide to adopt its functional 3D structure. This is consistent with the general notion that all of the chemical information needed for correct folding of proteins, including IMPs, is stored in their sequences.

From a more practical perspective, conditions initially established for BR refolding have been applied without much change to the folding of six distinct GPCRs, with functional yields between 30% and 70%. Should this approach turn out to be sufficiently general and easy to implement, as these data suggest, it would represent an important breakthrough for *in vitro* studies aimed at understanding the molecular basis of the function of rhodopsin-like GPCRs and possibly of many other IMPs. It should be noted in this context that all of the IMPs that have been folded to date using APols, despite differing in length (e.g. 248 aa for BR vs 472 aa for CB1), have relatively simple structures. In particular, none of them has extended complex extramembrane domains like the GPCRs from classes B (secretin receptor-like) and C (glutamate receptor-like) or from the adhesion and frizzled families. It is currently an open question whether APols would favor or interfere with the folding of such large extramembrane domains. It would be of interest to express and fold some reference GPCRs from classes B or C using the α_5 I-amphipol expression-folding strategy described above to assess whether the presence of a large soluble N terminus influences expression and functional folding. As indicated before, the class B GPCR GLP-1R has been accumulated in bacterial IBs and functionally folded by transfer from SDS to Brij78 in the presence of methyl- β -cyclodextrin [35]. However, the

Table 3. Applicability of various approaches for handling and studying GPCR–APol complexes

Approach	Applicability	Remarks	Refs
UV-visible absorption and fluorescence spectroscopy, CD	Yes, but for IR studies in the amide absorption bands	All APols validated to date contain amide bonds	[43,50,52]
Ligand-binding and functional studies	Yes	Damping of large-scale transmembrane conformational changes may occur. NAPols to be favored for G protein and arrestin binding studies, as well as for binding studies with cationic amphipathic ligands	[44,47,52,55,56,60,65]
Purification	Most purification techniques, including ultracentrifugation, size exclusion chromatography, immobilized metal and ligand-based affinity chromatography, etc.	With charged APols, ionic exchange chromatography and isoelectric focusing should be avoided	[44,50,52]
Electron microscopy, atomic force microscopy	Single particles studied by EM after negative staining and by cryoEM	No AFM studies reported yet	[18,52,59,66]
Immobilization onto a solid support	Yes	Immobilization can be either direct or mediated by an appropriately functionalized APol	[40,56]
NMR	Yes	Tested with A8-35, SAPols and NAPols, the latter two giving access to lower pH. Only A8-35 has been deuterated to date. No solid-state studies reported yet	[53–55,63]
Mass spectrometry	Yes	Study in progress	[54]
X-Ray crystallography	Remains to be developed	Transfer to detergent or lipid cubic phase	[18]

N-terminal domain of GLP-1R is rather short (predicted to be 122 aa). Trials to overexpress metabotropic glutamate receptors from the GPCR class C in *E. coli* IBs were not successful [27], but the α_5 I fusion strategy has not been applied to these targets so far. Scrambling of disulfide bridges has not been a problem until now, but will undoubtedly be in some cases. It might possibly be alleviated by careful control of the redox potential during and after folding, and/or by genetic engineering.

What to do next?

Once a GPCR has been folded using APols, it can be studied in this environment or transferred to another one. Most biochemical and biophysical techniques can be applied to APol-trapped MPs (Table 3) [18]. There are reasons to believe that studying protein–protein interactions such as GPCR oligomerization and recruitment of non-membrane protein partners from signaling complexes can be performed using APols. First, the binding of large soluble toxins and of antibodies to APol-trapped IMPs has already been described [40,56], and these data are promising for GPCRs and their signaling proteins. Second, GPCRs expressed in IBs and subsequently folded in detergent–lipid mixed micelles can be isolated as dimers and used to characterize molecular events that occur on activation [29,57,58]. In the same way, APol trapping does not prevent GPCRs from assembling into dimers [44]. Purified GPCRs can also interact functionally with signaling proteins. This is true for G proteins with receptors folded in mixed micelles [29,57,58], but also applies to receptors folded in APols. Indeed, both G proteins (G_s and G_q) and arrestins can bind to APol-trapped vasopressin V2 and ghrelin GHSR-1a GPCRs (J-L. Banères and B. Mouillac, unpublished data). Cryo-electron microscopy can be applied to APol-trapped IMP complexes [59] and supercomplexes (T. Althoff, PhD thesis, University of Frankfurt-am-Main, 2011), whose structure can then be solved by single-particle image analysis. This approach

could conceivably be used to study the arrangement of GPCRs associated into dimers (oligomers) and/or interacting with their associated signaling proteins. APol complexation is compatible with ligand binding studies [18,44,55,56,60], so trapping with an appropriately functionalized APol would provide a straightforward and very general approach to GPCR immobilization onto a solid support for ligand screening [56].

There are cases in which it might be desirable to transfer the folded GPCR to another environment. Direct transfer of IMPs from APols to lipid vesicles or black films has been demonstrated [43,61]. The procedure, however, is unlikely to be applicable to GPCRs, which are fragile proteins and are likely to be denatured in the process. A safer route would be to exchange the APol for detergent or lipid–detergent mixed micelles, which is readily possible [50,51,62], and then to proceed to a classical reconstitution.

Crystallization of APol-trapped IMPs is still in its infancy [18]. Crystallization of a GPCR that has been folded using APols should probably be attempted after receptor transfer to a detergent solution, to bicelles, to a lipidic cubic phase, etc. NMR seems to be a particularly promising route for studying the structure of ligands bound to APol-trapped GPCRs, ligand-induced conformational transitions and, possibly, at least some aspects of GPCR structure. Several small IMPs in complexes with APols have been studied to date using solution NMR [53,54,63,64]. GPCR–APol complexes, because of their large size, remain difficult to study *in toto*. However, transferred nuclear Overhauser effects (trNOEs) can be exploited to determine the structure of GPCR-bound ligands. In a recent study, a deuterated BLT2 receptor was folded and stabilized using a partially deuterated version of amphipol A8-35. One of its ligands, the leukotriene LTB₄, was then added in its hydrogenated form and the receptor-bound structure was determined from the magnitude of 89 trNOE signals [55]. The range of NMR studies applicable to APol-trapped GPCRs ought

to be extended by developments in the chemistry of APols, such as the availability of a perdeuterated version of A8-35 (F. Giusti, unpublished data) or of pH-insensitive APols such as SAPols (Figure 3b; T. Dahmane, PhD thesis, Université Paris 7, 2007) [47] and NAPols (Figure 3c; P. Bazzacco, PhD thesis, Université Paris 7, 2009) [48,49].

Acknowledgements

We would like to thank Drs T. Dahmane and L.J. Catoire for their critical reading of the manuscript.

References

- Lagerstrom, M.C. and Schioth, H.B. (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat. Rev. Drug Discov.* 7, 339–357
- Hofmann, K.P. et al. (2009) A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem. Sci.* 34, 540–552
- Rosenbaum, D.M. et al. (2009) The structure and function of G protein-coupled receptors. *Nature* 459, 356–363
- Warne, T. et al. (2008) Structure of a β_1 -adrenergic G protein-coupled receptor. *Nature* 454, 486–491
- Rasmussen, S.G. et al. (2007) Crystal structure of the human β_2 -adrenergic G protein-coupled receptor. *Nature* 450, 383–387
- Jaakola, V.P. et al. (2008) The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. *Science* 322, 1211–1217
- Wu, B. et al. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330, 1066–1071
- Chien, E.Y.T. et al. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* 330, 1091–1095
- Klammt, C. et al. (2007) Cell-free production of G protein-coupled receptors for functional and structural studies. *J. Struct. Biol.* 158, 482–493
- Reeves, P.J. et al. (2002) Structure and function in rhodopsin: a tetracycline inducible system in stable mammalian cell lines for high-level expression of opsin mutants. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13413–13418
- Mouillac, B. et al. (1992) Agonist-modulated palmitoylation of β_2 -adrenergic receptor in Sf9 cells. *J. Biol. Chem.* 267, 21733–21737
- Panneels, V. and Sinning, I. (2010) Membrane protein expression in the eyes of transgenic flies. *Methods Mol. Biol.* 601, 135–147
- Zhang, L. et al. (2005) Expression of functional G protein-coupled receptors in photoreceptors of transgenic *Xenopus laevis*. *Biochemistry* 44, 14509–14518
- Li, N. et al. (2007) Heterologous expression of the adenosine A1 receptor in transgenic mouse retina. *Biochemistry* 46, 8350–8359
- Sarramegna, V. et al. (2005) Solubilization, purification and mass spectrometry analysis of the human mu-opioid receptor expressed in *Pichia pastoris*. *Protein Expr. Purif.* 43, 85–93
- Lundstrom, K. (2005) Structural genomics of GPCRs. *Trends Biotechnol.* 23, 103–108
- Bokoch, M.P. et al. (2010) Ligand-induced regulation of the extracellular surface of a G protein-coupled receptor. *Nature* 463, 108–112
- Popot, J.-L. (2010) Amphipols, nanodiscs, and fluorinated surfactants: three nonconventional approaches to studying membrane proteins in aqueous solutions. *Annu. Rev. Biochem.* 79, 737–775
- Marullo, S. et al. (1988) Human β_2 -adrenergic receptors expressed in *Escherichia coli* membranes retain their pharmacological properties. *Proc. Natl. Acad. Sci. U.S.A.* 85, 7551–7555
- Tucker, J. and Grishammer, R. (1996) Purification of a rat neurotensin receptor expressed in *Escherichia coli*. *Biochem. J.* 317, 891–899
- Weiss, H.M. and Grishammer, R. (2002) Purification and characterization of the human adenosine A_{2a} receptor functionally expressed in *Escherichia coli*. *Eur. J. Biochem.* 269, 82–92
- Skretas, G. and Georgiou, G. (2009) Genetic analysis of G protein-coupled receptor expression in *Escherichia coli*. *Biotechnol. Bioeng.* 102, 357–367
- White, J.F. et al. (2004) Automated large-scale purification of a G protein-coupled receptor for neurotensin. *FEBS Lett.* 564, 289–293
- Kiefer, H. et al. (1996) Expression of an olfactory receptor in *Escherichia coli*: purification, reconstitution and ligand binding. *Biochemistry* 35, 16077–16084
- Banères, J.-L. et al. (2003) Structure-based analysis of GPCR function: conformational adaptation of both agonist and receptor upon leukotriene B4 binding to recombinant BLT1. *J. Mol. Biol.* 329, 801–814
- Banères, J.-L. et al. (2005) Molecular characterization of a purified 5-HT4 receptor. *J. Biol. Chem.* 280, 20253–20260
- Michalke, K. et al. (2009) Mammalian G protein-coupled receptor expression in *Escherichia coli*: I High throughput large-scale production as inclusion bodies. *Anal. Biochem.* 386, 147–155
- Michalke, K. et al. (2009) Mammalian G protein-coupled receptor expression in *Escherichia coli*: II Refolding and biophysical characterization of mouse cannabinoid receptor 1 and human parathyroid hormone receptor 1. *Anal. Biochem.* 401, 74–80
- Arcemisbèhère, L. et al. (2010) Leukotriene BLT2 receptor monomers activate the G_{i2} GTP-binding protein more efficiently than dimers. *J. Biol. Chem.* 285, 6337–6347
- Mouillac, B. and Banères, J.-L. (2010) Mammalian membrane receptors expression as inclusion bodies in *Escherichia coli*. *Methods Mol. Biol.* 601, 39–48
- Kiefer, H. (2003) In vitro folding of alpha-helical membrane proteins. *Biochim. Biophys. Acta* 1610, 57–62
- Huang, K.-S. et al. (1981) Refolding of an integral membrane protein Denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. *J. Biol. Chem.* 256, 3802–3809
- Muller, I. et al. (2008) The full-length mu-opioid receptor: a conformational study by circular dichroism in trifluoroethanol and membrane-mimetic environments. *J. Membr. Biol.* 223, 49–57
- Miller, D. et al. (2009) In vitro unfolding and refolding of the small multidrug transporter EmrE. *J. Mol. Biol.* 393, 815–832
- Schröder-Tittmann, K. et al. (2010) Recombinant expression, in vitro refolding, and biophysical characterization of the human glucagon-like peptide-1 receptor. *Biochemistry* 49, 7956–7965
- Popot, J.-L. et al. (1987) Refolding of bacteriorhodopsin in lipid bilayers. A thermodynamically controlled two-stage process. *J. Mol. Biol.* 198, 655–676
- Schmidt, P. et al. (2010) A reconstitution protocol for the in vitro folded human G protein-coupled Y2 receptor into lipid environment. *Biophys. Chem.* 150, 29–36
- Schimmer, S. et al. (2010) Functional characterization of the in vitro folded human Y1 receptor in lipid environment. *Protein Pept. Lett.* 17, 605–609
- Tribet, C. et al. (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions. *Proc. Natl. Acad. Sci. U.S.A.* 93, 15047–15050
- Popot, J.-L. et al. (2003) Amphipols: polymeric surfactants for membrane biology research. *Cell. Mol. Life Sci.* 60, 1559–1574
- Gohon, Y. and Popot, J.-L. (2003) Membrane protein-surfactant complexes. *Curr. Opin. Colloid Interface Sci.* 8, 15–22
- Sanders, C.R. et al. (2004) French swimwear for membrane proteins. *ChemBioChem.* 5, 423–426
- Pocanschi, C.L. et al. (2006) Amphipathic polymers: tools to fold integral membrane proteins to their active form. *Biochemistry* 45, 13954–13961
- Dahmane, T. et al. (2009) Amphipol-assisted in vitro folding of G protein-coupled receptors. *Biochemistry* 48, 6516–6521
- Gohon, Y. et al. (2004) Partial specific volume and solvent interactions of amphipol A8-35. *Anal. Biochem.* 334, 318–334
- Gohon, Y. et al. (2006) Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8-35. *Langmuir* 22, 1281–1290
- Picard, M. et al. (2006) Protective and inhibitory effects of various types of amphipols on the Ca^{2+} -ATPase from sarcoplasmic reticulum: a comparative study. *Biochemistry* 45, 1861–1869
- Sharma, K.S. et al. (2008) Glucose-based amphiphilic telomers designed to keep membrane proteins soluble in aqueous solutions: synthesis and physicochemical characterization. *Langmuir* 24, 13581–13590
- Bazzacco, P. et al. (2009) Trapping and stabilization of integral membrane proteins by hydrophobically grafted glucose-based telomers. *Biomacromolecules* 10, 3317–3326

- 50 Zoonens, M. *et al.* (2007) Dynamics of membrane protein/amphipol association studied by Förster resonance energy transfer: Implications for in vitro studies of amphipol-stabilized membrane proteins. *Biochemistry* 46, 10392–10404
- 51 Tribet, C. *et al.* (2009) Thermodynamic characterization of the exchange of detergents and amphipols at the surfaces of integral membrane proteins. *Langmuir* 25, 12623–12634
- 52 Gohon, Y. *et al.* (2008) Bacteriorhodopsin/amphipol complexes: structural and functional properties. *Biophys. J.* 94, 3523–3537
- 53 Zoonens, M. *et al.* (2005) NMR study of a membrane protein in detergent-free aqueous solution. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8893–8898
- 54 Catoire, L.J. *et al.* (2009) Inter- and intramolecular contacts in a membrane protein/surfactant complex observed by heteronuclear dipole-to-dipole cross-relaxation. *J. Magn. Res.* 197, 91–95
- 55 Catoire, L.J. *et al.* (2010) Structure of a GPCR ligand in its receptor-bound state: leukotriene B4 adopts a highly constrained conformation when associated to human BLT2. *J. Am. Chem. Soc.* 132, 9049–9057
- 56 Charvolin, D. *et al.* (2009) The use of amphipols as universal molecular adapters to immobilize membrane proteins onto solid supports. *Proc. Natl. Acad. Sci. U.S.A.* 106, 405–410
- 57 Damian, M. *et al.* (2006) Asymmetric conformational changes in a GPCR dimer controlled by G proteins. *EMBO J.* 25, 5693–5702
- 58 Damian, M. *et al.* (2008) G protein activation by the leukotriene B4 receptor dimer. Evidence for an absence of trans-activation. *J. Biol. Chem.* 283, 21084–21092
- 59 Flötenmeyer, M. *et al.* (2007) The use of amphipathic polymers for cryo-electron microscopy of NADH:ubiquinone oxidoreductase (complex I). *J. Microsc.* 227, 229–235
- 60 Martinez, K.L. *et al.* (2002) Allosteric transitions of *Torpedo* acetylcholine receptor in lipids, detergent and amphipols: molecular interactions vs. physical constraints. *FEBS Lett.* 528, 251–256
- 61 Nagy, J.K. *et al.* (2001) Use of amphipathic polymers to deliver a membrane protein to lipid bilayers. *FEBS Lett.* 501, 115–120
- 62 Tribet, C. *et al.* (1997) Stabilisation of hydrophobic colloidal dispersions in water with amphiphilic polymers: application to integral membrane proteins. *Langmuir* 13, 5570–5576
- 63 Catoire, L.J. *et al.* (2010) Solution NMR mapping of water-accessible residues in the transmembrane β -barrel of OmpX. *Eur. Biophys. J.* 39, 623–630
- 64 Raschle, T. *et al.* (2010) Nonmicellar systems for solution NMR spectroscopy of membrane proteins. *Curr. Opin. Struct. Biol.* 20, 1–9
- 65 Gorzelle, B.M. *et al.* (2002) Amphipols can support the activity of a membrane enzyme. *J. Am. Chem. Soc.* 124, 11594–11595
- 66 Tribet, C. *et al.* (1998) Scanning transmission electron microscopy study of the molecular mass of amphipol/cytochrome *b₆f* complexes. *Biochimie* 80, 475–482