Chapter 1
Membrane Protein Production for Structural Analysis

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1.1 Introduction

Integral membrane proteins (IMPs) account for roughly 30% of all open reading frames in fully sequenced genomes (Liu and Rost 2001). These proteins are of main importance to living cells. They are involved in fundamental biological processes like ion, water, or solute transport, sensing changes in the cellular environment, signal transduction, and control of cell–cell contacts required to maintain cellular homeostasis and to ensure coordinated cellular activity in all organisms. IMP dysfunctions are responsible for numerous pathologies like cancer, cystic fibrosis, epilepsy, hyperinsulinism, heart failure, hypertension, and Alzheimer diseases. However, studies on these and other disorders are hampered by a lack of information about the involved IMPs. Thus, knowing the structure of IMPs and understanding their molecular mechanism not only is of fundamental biological interest but also holds great potential for enhancing human health. This is of paramount importance in the pharmaceutical industry, which produces many drugs that bind to IMPs, and recognizes the potential of many recently identified G-protein-coupled receptors (GPCRs), ion channels, and transporters, as targets for future drugs. GPCR, which account for 50% of all drug targets, is one of the largest and most diverse IMP families encoded by more than 800 genes in the human genome (Fredriksson et al. 2003; Lundstrom 2006). However, whereas high-resolution structures are available for a myriad of soluble proteins (more than 42,000 in the Protein Data Bank,

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PDB), atomic structures have so far been obtained for only 474 IMPs, with 150 new structures determined in 2012 and 2013 (see http://blanco.biomol.uci.edu/mpstruc/), but only 10 % of the unique IMP structures are derived from vertebrates. The first mammalian IMPs were crystallized on to their natural abundance, circumventing all the difficulties associated with overexpression (ATP synthase, Stock et al. 1999; rhodopsin, Palczewski et al. 2000; and Calcium ATPase, Toyoshima et al. 2000). However, the majority of medically and pharmaceutically relevant IMPs are present in tissues at very low concentration, making production of recombinant IMPs in heterologous systems suitable for large-scale production a prerequisite for structural studies. In 2005, the two first atomic structures of recombinant mammalian IMPs were obtained from proteins overexpressed in yeast: the calcium ATPase from sarcoplasmic reticulum SERCA1A in *Saccharomyces cerevisiae* (Jidenco et al. 2005) and the Kv1.2 voltage-gated potassium channel in *Pichia pastoris* (Long et al. 2005). Since then, extensive optimization of heterologous expression systems (Mus-Veteau 2010) has begun to bear fruit, and early 2014 the structure of 37 recombinant mammalian IMPs were determined, of which 20 belong to GPCRs (Table 1.1; Venkatakrishnan et al. 2013). Impressive progress has been made in the 2012 and 2013 with more than 21 new structures of recombinant mammalian IMPs determined, 13 belonging to GPCRs. The large majority of these structures were obtained from IMPs overexpressed in Sf9 insect cells using recombinant baculovirus (21 proteins over the 37, 14 being GPCRs). From IMPs produced in the yeast ten structures were obtained from Pichia pastoris and one from *Saccharomyces cerevisiae*, three were determined from proteins produced in the bacteria *Escherichia coli,* two from proteins expressed in mammalian cells. Concurrently, with the advances in recombinant mammalian IMP production, improvement of the stabilization strategies of IMPs in solution has contributed to the growing number of IMP structures solved. Indeed, purification of IMPs requires the use of detergents to extract IMPs from membrane and to maintain them in aqueous solution (in complex with detergents and lipids). Many mammalian IMPs are unstable in detergent solution, and finding suitable detergent and conditions that ensure protein homogeneity, functionality, stability, and crystallization is often a limiting and crucial step (Tate 2010). New surfactants able to maintain IMPs in solution with less denaturing effect have been synthesized and are currently under development (Chap. 7 by Zoonens et al. and Chap 8 by Durand et al. in this volume). Among these new surfactants, Maltose-neopentyl glycol (MNG; Chae et al. 2010) allowed the crystallization and structure determination of δ (Manglik et al. 2012) and μ (Granier et al. 2012) opioid receptors. Another strategy that has been shown to be highly efficient to stabilize IMPs in solution is protein engineering. For the majority of the structures solved, N- and C-terminus, which are usually flexible or not ordered structures that can prevent crystallization, have been truncated (Table 1.1). For GPCRs which are highly instable in solution, truncation of N- and C-terminal domains were not sufficient, additional strategies were necessary to stabilize these proteins: (1) the replacement of a flexible loop by a stable soluble protein domain such as T4 lysozyme (T4L) or apocytochrome b_{562} RIL has been a successful strategy for the determination of a dozen of IMP structures, (2) thermostabilizing point mutations, (3) engineering

disulfide bridges and *N*-glycosylation sites, or (4) a mixture of these strategies, e.g., serotonin receptor and smoothened structures were obtained using apocytochrome b_{562} RIL and thermostabilizing point mutations (Wacker et al. 2013; Wang et al. 2013); chemokine receptor CXCR4 (Wu et al. 2010), dopamine D3 receptor (Chien et al. 2010), and neurotensin receptor (White et al. 2012) structures were obtained using T4L and thermostabilizing point mutations; and the nociceptin/orphanin receptor (NOP) structure was solved using both apocytochrome b_{562} RIL and T4L fusions (Table 1.1; Thompson et al. 2012). Often, it was necessary to use ligands, agonists, or antagonists to enhance receptor stability. Improvement in structural analysis techniques for IMPs also contributed to the exponential growth of the number of mammalian IMP atomic structures observed these past few years. X-ray crystallography is the technique which let the resolution of the most structures, and advances in micro-crystallography have allowed obtaining higher-resolution diffraction from smaller crystals (Moukhametzianov et al. 2008). Vapor diffusion with hanging drops is the most commonly used crystallization strategy. However, use of lipidic cubic phase (LCP; Caffrey 2009) and new detergents such as MNG (Chae et al. 2010) has improved the likelihood of obtaining crystals. This strategy was applied for most of the GPCR structures. Electron diffraction allowed the structure resolution at atomic level of only one mammalian recombinant IMP, the aquaporin AQP4 (Hiroaki et al. 2005; Tani et al. 2009). Recent progresses in solution and solid-state nuclear magnetic resonance (ssNMR) methods (Maslennikov and Choe, 2013) have permitted the determination of the atomic structure of three mammalian recombinant IMPs: the chemokine receptor CXCR1 (Park et al. 2012), the mitochondrial uncoupling protein UCP2 (Berardi et al. 2011), and the phospholamban (Oxenoid and Chou 2005; Verardi et al. 2011).

Table 1.1 reports the strategies that allowed determination of recombinant mammalian IMPs. Lots of tools and strategies in the field of heterologous expression systems, stabilization, and structural analyses are still under development. This chapter introduces the tools developed in the past few years to increase the number of atomic structure of recombinant mammalian IMPs.

1.2 Production of Recombinant IMPs

It is clear that the first and probably the narrowest bottleneck in IMP expression is the production of abundant quantity of material. It is true, indeed, that the majority of structures for vertebrate IMPs were solved from native material and not from recombinant ones (Stock et al. 1999; Palczewski et al. 2000; Toyoshima et al. 2000). On the other hand, the larger part of medically and pharmaceutically relevant MPs is found at very low concentration, thus rendering overexpression of recombinant MPs essentials for large-scale production for structural studies. In the recent years, the panel of possibilities for overexpression of IMPs has become larger and larger, from *E. coli* to insect and mammalian cells passing by yeast systems and *in vitro* production, in order to create the "right expression system" for each protein. Indeed,

IMPs are very different in structure and physical–chemical properties, thus making it difficult to predict the good approach. In any case, each system has pros and cons, and the choice is often empirical, especially with regard to the levels of functional protein expression. In other words, the more we have the better it is.

The most widely used organism is still *E. coli* (for a review, see Sahdev et al. 2008), and since it presents disadvantages of improper folding with inclusion body (IB) formation, up-to-date efforts are concentrated to create new strains able to allow improved control on protein expression. The best-known strains are probably those of Miroux (Miroux and Walker 1996; see also for a review, Chap. 4 by Hattab et al. in this volume) that display internal membrane proliferation in which all the overexpressed proteins are located (Arechaga 2000). The results from Walker's laboratory still constitute the basement for engineering new strains. This is the case of the Lemo21(DE3) strain (Schlegel et al. 2012) in which the modulation of the activity of the T7 RNAP by the T7 lysozyme is the key to optimize the ratio of IMPs properly inserted in the cytoplasmic membrane to noninserted proteins. In this strain, maximizing the yields of IMPs is accompanied by reduction of the harmful effects of MP overexpression, resulting in stable overexpression. Moreover, IMPs produced in Lemo21(DE3) can be used for functional and structural studies demonstrating that the overexpressed material is not only inserted in the cytoplasmic membrane but also properly folded.

Another approach, which seems promising for eukaryotic IMPs, relies on new fusion protein expression of the amphipathic Mistic protein as a cargo to drive IMPs to the membrane (Roosild et al. 2005). This strategy was used to express the chloroplast ATP/ADP transporter from *Arabidopsis thaliana* (NTT1) and characterize its transport properties (Deniaud et al. 2011). NTT1 fused to Mistic has a very low transport activity, which can be recovered after *in vivo* Mistic fusion cleavage. Therefore, if one considers the high yield of mature NTT1 obtained via the Mistic fusion approach, this becomes a valid approach for obtaining quantities of pure and active proteins that are adequate for structural studies.

Besides *E.coli,* there are other bacteria that seem to be well adapted to IMP overexpression, such as *Lactococcus lactis* and *Rhodobacter sphaeroides*. The first one is a nonpathogenic and noninvasive lactic acid Gram-positive bacterium (for a review, see Junge et al. 2008; Frelet-Barrand et al. 2010a, 2010b; see also Chap. 5 in this volume). The recombinant proteins are expressed under the control of the Nisin-inductible promoter (NICE system), and the access to new technology as the gateway one renders possible efficient cloning strategies. Recently, it has been shown that the Mistic fusion can be used to facilitate high-yield production also in *L. lactis* (Xu et al. 2013).

The prokaryotic systems we have described above are the ones giving the largest amount of recombinant proteins, even if often not in a functional form, since post-translational modifications are needed. Many eukaryotic IMPs are indeed unstable during purification and detergent manipulation even if the barrier of the poor overexpression can be overcome with generic strategies (Bill et al. 2011). Moreover, there are bacteria able to overcome this step, as an *E. coli* strain that allows glyco-

sylation (Chen et al. 2012), but nevertheless it is often necessary to turn to eukaryotes to assure the best ratio of produced versus functional protein.

The tendency is now to use systems which are closer and closer to the native environment of the protein we want to express: yeast, plant, insect, and mammalian cells seem to trace an approach route to the most effective strategy.

Yeasts are able to perform various post-translational modifications including proteolytic processing of signal sequences, disulfide-bond formation, acylation, prenylation, phosphorylation, and certain types of glycosylation that are crucial for activity and folding. Although the lipid composition of yeast membranes is similar to higher eukaryotes, the absence of specific sterols, e.g., cholesterol for mammalian proteins, might affect protein functionality. Ergosterol, the predominant sterol in yeast, compensates for some mammalian proteins, but for full activity, presence of cholesterol might be essential (Tate et al. 1999). *S. cerevisiae* has been largely employed for eukaryotic MPs due to the well-known genetic composition, the large number of available strains, and the facility to tune expression via inducible promoters. Nevertheless, *S. cerevisiae* is not easy to handle in fermenter condition, and for this reason, attention has been focused on other yeasts such as *P. pastoris* that can reach high cell density and have the same "good heterologous MP producer potential" as *S. cerevisiae.* Quite a lot of efforts are now concentrated to decipher the physiological response of yeasts to MPs' overexpression. To this end, Bonander et al. (2005) studied the impact of pH and temperature on the expression of Fps1p, a glycerol uptake/efflux facilitator, and they demonstrated that optimal conditions for growing are not always profitable for functional, overexpressed MPs. Optimizing culture conditions to the end of MPs' production seems to be a real opportunity for both *P. pastoris* and *S. cerevisiae* (for a review Bonander and Bill 2012).

Going upward to the "best adapted" expression system, it is easy to run into baculovirus/insect cell expression system, which is a kind of compromise between bacterial and mammalian cells. This system is well adapted for eukaryotic proteins because of similar codon usage, better expression levels, and fewer truncated proteins. Moreover, insect cells allow post-translational modifications that are closer to those of mammalian cells than those produced by bacteria or even yeast (Jarvis and Finn 1995). Briefly, the baculovirus system relies on the infection of insect cell lines (usually Sf9, Sf2) by recombinant viruses encoding the gene(s) of interest. Improvements in recombinant baculovirus generation have been implemented over the past two decades (Condreay and Kost 2007), including a system (BacMam, Invitrogen) which allows baculovirus-based expression in mammalian cells.

Recently, structures of mammalian IMPs as bovine rhodopsin (Standfuss et al. 2007; Standfuss et al. 2011; Deupi et al. 2012) and the human ammonia transporter RhCG (Gruswitz et al. 2010) have been determined upon overexpression in mammalian cells (Table 1.1). Different approaches using transient or stables cell lines could be used, the first being relatively rapid to settle and the second much longer. Moreover, the choice of the promoter—inducible or constitutive—together with the choice of the cell line is critical for the good issue of the study (Andrell and Tate 2013).

A paradigm of the importance to find the right expression system has been described for the serotonin transporter (SERT; Tate et al. 2003). The most detailed

study on different expression systems on a unique protein, indeed, is the one on SERT. This protein needs glycosylation to be correctly folded in the presence of calnexin (Tate et al. 1999), and has a strict requirement for cholesterol. For evident reasons, *E. coli* and yeast are inappropriate to overexpress SERT, and insect cells, even though they have the entire requirement for a correct expression, led to an inactive protein (Tate et al. 1999). The same group turned the effort to mammalian systems (Tate el al. 2003), and they succeeded with the tetracycline-inducible system (Andrell and Tate 2013).

Last but not the least, cell-free (CF) systems are evolving as a valid alternative in IMP expression. The most classical extracts from *E. coli* and wheat germ are used routinely for structural approaches as they tolerate additives for the co-translational solubilization of CF system-expressed IMPs. The palette of molecules for protein solubilization in CF systems is now very varied and is increased by the possibility of compounds mixture (Park et al. 2011; Bazzacco et al. 2012; Junge et al. 2010; Ma et al. 2011). A valid alternative to tensioactifs is the insertion in lipid bilayer or nanodiscs (Periasamy et al. 2012; Roos et al. 2012). In the past years, CF system-produced MPs have been used for structural studies, particularly NMR spectroscopy as the case of the C-terminal fragment of human presenilin-1, a subunit of the γ-secretase complex (Sobhanifar et al. 2010b). CF systems also allow the expression and the structural evaluation of membrane complexes such as ATP synthase, which results in a fully assembled complex in the CF system in the presence of detergents (Matthies et al. 2011). CF system-expressed MPs also gave some promising results in crystallization: the human voltage-dependent anion channel-1 structure was solved at low resolution (Deniaud et al. 2010) and the structure of a eukaryotic rhodopsin was solved after *in vitro* production in the presence of a mixture of lipids and detergents (Wada et al. 2011). CF systems are then a powerful approach to produce difficult proteins such IMPs, and their development passes through efficient CF extract sources, which are essential for the preparative-scale CF system production of post-translationally modified proteins (for details, see Chap. 2 by Proverbio et al. in this volume).

In conclusion overexpression of IMPs is, in a sense, fighting against evolution since most IMPs have not evolved to be abundant a few thousand copies per cell. Is that the reason why we are obliged to circumvent the bottleneck and look for new methodology strategies? As we said before, “the more we have the better it is” seems to be a good sentence…

1.3 Stabilization of Solubilized IMPs for Structural Analysis

Aqueous solubilization, necessary for structural analysis, generally requires a detergent to shield the large lipophilic surfaces displayed by IMPs. Unfortunately, IMPs tend to denature, aggregate, or remain unstable in detergents. The poor stability of the detergent-solubilized IMP in a form that is amenable for crystal formation

Table 1.1 Recombinant mammalian IMPs for which the structure has been solved

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
G protein-coupled receptors							
Rhodopsin (two structures)							
N2C/D282C mutant		*Bos taurus*	COS cells	Disulfide bond between the N-terminus and loop E3 (enhanced thermostability: 10 °C)	C8E4	X-ray: vapor diffusion and sitting drops *3,4 Å*	Standfuss et al. (2007)
M257Y mutant	Constitutively active meta-II state, in complex with GαCT	*B. taurus*	HEK2935-GnTl-cells	Constitutively active mutation and agonist peptide GαCT	OG	X-ray: vapor diffusion and sitting drops *3.30 Å*	Deupi et al. (2012)
β1 adrenergic receptor (5 structures)							
β1AR36-m23: removal of flexible regions at the N and C terminus, and in the cytoplasmic loop 3, 8 point mutations	Bound to antagonist cyanopindolol	Turkey	Insect cells infected by baculovirus	Six point mutations (enhanced thermostability: 21 °C) and antagonist	OG	X-ray: vapor diffusion/hanging drops *2.7 Å*	Warne et al. (2008)
β1AR36-m23	Bound to agonists carmotero/isoprenalin	Turkey	Insect cells infected by baculovirus	Six point mutations (enhanced thermostability: 21 °C) and agonist	Hega-10 and CHS	X-ray: vapor diffusion/hanging drops *2.50 Å*	Warne et al. (2011)
β1AR36-m23	Inactive state bound to antagonist carazolol	Turkey	Insect cells infected by baculovirus	Six point mutations (enhanced thermostability: 21 °C) and antagonist	Hega-10	X-ray: vapor diffusion/hanging drops *3.00 Å*	Moukhametzianov et al. (2011)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
β1AR36-m23	Bound to carvedilol (a biased agonist and beta blocker)	Turkey	Insect cells infected by baculovirus	Six point mutations (enhanced thermostability: 21 °C) and antagonist	Hega-10	X-ray: vapor diffusion/sitting drops *2.30 Å*	Warne et al. (2012)
β1 adrenergic receptor oligomer	Ligand-free basal state in a lipid membrane-like environment	Turkey	Insect cells infected by baculovirus		DDM + lipids POPC/POPE/POPG/ cholesterol	X-ray: vapor diffusion/hanging drops *3.50 Å*	Huang et al. (2013)
β2 adrenergic receptor (seven structures)							
β2AR365-Fab5 complex	Bound to an inverse agonist	*Homo sapiens*	Insect cells infected by baculovirus	Increasing the polar surface by interaction with Fab5 from monoclonal antibody	Bicelles composed of the lipid DMPC and the detergent CHAPSO	X-ray: vapor diffusion/hanging drops *3.4/3.7 Å*	Rasmussen et al. (2007)
Methylated β2AR365-Fab5 complex		*H. sapiens*	Insect cells infected by baculovirus	Interaction with Fab5	DDM + CHS	X-ray and NMR spectroscopy *3.4 Å*	Bokoch (2010)
β2AR-T4L: T4L replaces third intracellular loop	Bound to the partial inverse agonist carazolol	*H. sapiens*	Insect cells infected by baculovirus	Cholesterol		X-ray with cholesterol-doped lipid cubic phase (LCP) *2.4 Å*	Cherezov et al. (2007)
β2AR-E122W-T4L: C-terminus truncated	Bound to cholesterol and partial inverse agonist timolol	*H. sapiens*	Insect cells infected by baculovirus	Cholesterol and the partial inverse agonist timolol		X ray with LCP *2.8 Å*	Hanson et al. (2008)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
β2AR-T4L	In complex with a "novel inverse agonist"	*H. sapiens*	Insect cells infected by baculovirus			X-ray with LCP *2.84 Å*	Wacker et al. (2010)
β2AR-T4L	Agonist-bound active state	*H. sapiens*	Insect cells infected by baculovirus	Nanobody and agonist BI-167107		X-ray: LCP with 7.7 MAG containing 10% cholesterol *3.50 Å*	Rasmussen et al. (2011)
β2AR-T4L-H93C with agonist covalently linked by a disulfide bond	Agonist-bound active state	*H. sapiens*	Insect cells infected by baculovirus	Agonist covalently linked		X-ray with cholesterol-doped monoolein cubic phase *3.50 Å*	Rosenbaum et al. (2011)
β2AR-Gs protein complex with T4L fused to the amino terminus of the β2AR	Active state ternary complex: agonist/monomeric β(2)AR/nucleotide-free Gs heterotrimer	*H. sapiens*	Insect cells infected by baculovirus	Nanobody (Nb35) that binds at the interface between the Gα and Gβ subunits		X ray: LCP with MAG7.7 *3.20 Å*	Rasmussen et al. (2011)
A_{2A} adenosine receptor (six structures)							
A_{2A}AR-T4L-ΔC: T4L inserted between TM V and VI	In complex with a high-affinity subtype-selective antagonist ZM241385	*H. sapiens*	Insect cells infected by baculovirus			X-ray: LCP with monoolein, and cholesterol *2.6 Å*	Jaakola et al. (2008)
A_{2A}AR-T4L-ΔC	With bound agonist (UK-432097)	*H. sapiens*	Insect cells infected by baculovirus			X-ray: LCP with monoolein, and cholesterol *2.71 Å*	Xu et al. (2011)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
A_{2A}AR-GL31 with four thermostabilising point mutations (L48A, A54L, T65A and Q89A3) and N-glycosylation site mutation N154A	Intermediate conformation between the inactive and active states with bound adenosine	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilising mutations	OG + CHS	X-ray: vapor diffusion *3.00 Å*	Lebon et al. (2011)
A_{2A}-StaR2: A_{2A}R-GL31, 8 thermostabilising mutations, truncation of the C-terminus	Inactive state conformation in complex with caffeine	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilising mutations A54 L, T88A, R107A, K122A, L202A, L235A, V239A, S277A	NG	X-ray: vapor diffusion *3.60 Å*	Doré et al. (2011)
$A_{2A}AR^{N154Q}$ with N-glycosylation site mutation N154A	In complex with inverse-agonist and antibody	*H. sapiens*	*Pichia pastoris*	Fab-fragment and antagonist (ZM241385)	OG	X-ray: vapor diffusion/hanging drops *2.70 Å*	Hino et al. (2012)
A_{2A}AR-BRIL-ΔC: third intracellular loop replaced by apocytochrome b(562)RIL	In complex with a high-affinity subtype-selective antagonist ZM241385	*H. sapiens*	Insect cells infected by baculovirus	Apocytochrome b562RIL (BRIL)		X ray: LCP with monoolein and cholesterol *1.80 Å*	Liu et al. (2012a)
CXCR1 chemokine receptor							
$^{13}C/^{15}N$-labeled full-length GST–CXCR1	Active receptor reconstituted in phospholipid bilayers and bound to interleukin-8 (IL-8)	*H. sapiens*	*Escherichia coli*	Refolding in DMPC proteoliposomes		Magic angle spinning (MAS) and oriented-sample (OS) solid-state NMR	Park et al. (2012)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
CXCR4 chemokine receptor							
Replacement of the third cytoplasmic loop (ICL3) with T4L and thermostabilizing L125W mutation	Complexed with IT1t antagonist	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilizing L125W mutation		X-ray: LCP with monoolein, and cholesterol *2.5 Å*	Wu et al. (2010)
Dopamine D3 receptor							
D3R-T4L: replacement of the third cytoplasmic loop (ICL3) with T4L and thermostabilizing mutation L119W	Complexed with D2/D3-selective antagonist	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilizing mutation L119W and antagonist eticlopride		X-ray: LCP with monoolein, and cholesterol *2.89 Å*	Chien et al. (2010)
Histamine H1 receptor							
H1R-T4L: replacement of the third cytoplasmic loop with T4L and deletion of 19 N-terminal residues-terminal residues	Complexed with antagonist doxepin	*H. sapiens*	*P. pastoris*			X-ray: LCP with monoolein, and cholesterol *3.10 Å*	Shimamura et al. (2011)
Sphingosine 1-phosphate receptor							
S1P1-T4L: replacement of the third cytoplasmic loop with T4L	Complexed with an antagonist sphingolipid mimic	*H. sapiens*	Insect cells infected by baculovirus		DDM + CHS	X-ray: LCP with monoolein, and cholesterol *3.35 Å*	Hanson et al. (2012)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
M2 muscarinic acetylcholine receptor							
M2-T4L: replacement of the third cytoplasmic loop with T4L, mutation of the N-linked glycosylation sites	Bound to an antagonist	*H. sapiens*	Insect cells infected by baculovirus	MNG	MNG	X-ray: LCP with monoolein, and cholesterol *3.00 Å*	Haga et al. (2012)
M3 muscarinic acetylcholine receptor							
M3-T4 L: replacement of the third cytoplasmic loop with T4L	In complex with tiotropium (Spiriva), a potent muscarinic inverse agonist	*Rattus norvegicus*	Insect cells infected by baculo virus	MNG and inverse agonist tiotropium	MNG	X-ray: LCP with monoolein, and cholesterol *3.40 Å*	Kruse et al. (2012)
κ-opioid receptor							
hKOR-T4 L: replacement of the third cytoplasmic loop with T4L	In complex with the antagonist JDTic	*H. sapiens*	Insect cells infected by baculovirus			X-ray: LCP with monoolein, and cholesterol *2.90 Å*	Wu et al. (2012)
μ-opioid receptor							
μOR-T4L: replacement of the third cytoplasmic loop with T4L, carboxy terminus truncated	Bound to a morphinan antagonist	*Mus musculus*	Insect cells infected by baculovirus	MNG + CHS	MNG + CHS	X-ray: LCP with monoolein, and cholesterol *2.80 Å*	Manglik et al. (2012)
δ-opioid receptor							
δ-OR-T4L: replacement of the third cytoplasmic loop with T4L	In complex with the antagonist naltrindol	*M. musculus*	Insect cells infected by baculovirus	MNG + CHS + antagonist naltrindol	MNG + CHS	X-ray: LCP with monoolein, and cholesterol *3.40 Å*	Granier et al. (2012)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
Nociceptin/orphanin FQ (N/OFQ) receptor							
BRIL-NOP-T4L: replacement of the third cytoplasmic loop with T4L, of N-terminus with b562RIL (BRIL) and truncation of 31 C-terminal residues-terminal residues	In complex with the peptide mimetic antagonist C-24	*H. sapiens*	Insect cells infected by baculovirus	Replacement of N-terminus with b_{562}RIL (BRIL) and addition of antagonist C-24		X-ray: LCP with monoolein, and cholesterol *3.01 Å*	Thompson et al. (2012)
NTS1 neurotensin receptor							
NTS1-GW5-T4L: replacement of the third cytoplasmic loop with T4L and 5 thermostabilizing point mutations	Bound to the C terminal portion (NT8-13) of the endogenous agonist neurotensin	*R. norvegicus*	Insect cells infected by baculovirus	Six stabilizing mutations; A86L, E166A, G215A, L310A, F358A, V360A, and addition of the agonist NT, MNG + CHS		X-ray: LCP with monoolein, and cholesterol *2.80 Å*	White et al. (2012)
Protease-activated receptor 1 (PAR1)							
PAR1-T4L: replacement of the third cytoplasmic loop with T4L, mutation of the N-linked glycosylation sites in ECL2 and truncation of the N- and C- terminus	Bound with antagonist vorapaxar	*H. sapiens*	Insect cells infected by baculovirus	Antagonist vorapaxar	DDM, CHS and sodium cholate	X-ray: LCP with monoolein, and cholesterol *2.20 Å*	Zhang et al. (2012)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
5-HT1B serotonin receptor							
5HT1B-BRIL: b562 RIL (BRIL) replaces cytoplasmic loop 3, truncation of the N-terminus to remove all glycosylation sites, and point mutation L138W	With bound ergotamine	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilizing mutation L138W and addition of ergotamine	DDM + CHS	X-ray: LCP with monoolein, and cholesterol *2.70 Å*	Wacker et al. (2013)
5-HT2B serotonin receptor							
5-HT2B-BRIL: b562 RIL (BRIL) replaces cytoplasmic loop 3, truncation of the N- and C- terminus, and introduction of M144W mutation	With bound ergotamine	*H. sapiens*	Insect cells infected by baculovirus	Thermostabilizing mutation M144W and addition of ergotamine	DDM + CHS	X-ray: LCP with monoolein, and cholesterol *2.70 Å*	Wacker et al. (2013)
Smoothened (SMO) receptor							
BRIL–ΔCRD–SMO–ΔC: b562 RIL and truncation of N and the C terminus	With bound antagonist LY2940680	*H. sapiens*	Insect cells infected by baculovirus	Antagonist LY2940680	DDM + CHS	X-ray: LCP with monoolein, and cholesterol *2.45 Å*	Wang et al. (2013)
Channels: potassium and sodium ion-selective							
Two-pore domain potassium channel							
K2P1.1 (TWIK-1)		*H. sapiens*	*P. pastoris*			*3.40 Å*	Miller and Long (2012)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
Voltage-gated potassium channel							
Kv1.2	Open in complex with an oxido-reductase beta subunit	*R. norvegicus*	*P. pastoris*			*2.9 Å*	Long et al. (2005) Chen et al. (2010)
Kv1.2/Kv2.1 voltage-gated potassium channel chimera							
WT and F233W voltage-sensor paddle transferred from Kv2.1 to Kv1.2, His10 tag at the amino terminus. Co-expressed with the rat β2.1 subunit	The pore is open and the voltage sensors adopt a membrane depolarized conformation	*R. norvegicus*	*P. pastoris*		Cymal-6, Cymal-7, CHAPS and lipids (POPC, POPE and POPG)	X-ray: vapor diffusion/hanging drops *2.4 Å* and *2.9 Å*	Long et al. (2007); Tao et al. (2010)
Two-pore domain potassium channel K2P4.1 (TRAAK)							
Codon-optimized, two consensus N-linked glycosylation sites mutated (N104Q, N108Q) and truncation of the C-terminal EGFP and 10xHistag in C-terminus		*R. norvegicus*	*P. pastoris*		DDM + Fos-choline 12	X-ray: vapor diffusion/hanging drops *3.80 Å*	Brohawn et al. (2012, 2013)
Inward-rectifier potassium channel							
Kir2.2 Synthetic gene from 38 to 369 with GFP and 1D4 antiboby recognition sequence in C-terminus	Without substrate In complex with PIP2	*Gallus gallus*	*P.* pastoris		DM	X-ray: vapor diffusion/hanging drops *3.1 Å* *3.31 Å*	Tao et al (2009); Hansen et al. (2011)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
G protein-gated inward rectifying potassium channel GIRK2							
Kir3.2 Deletion of N- and C-termini, EGFP and 10xHistag in C-terminus Wild-type and constitutively active mutant	Close and open conformation in complex with PIP2 In complex with βγ G-protein subunits: pre-open state intermediate between closed and open conformation	*M. musculus*	*P. pastoris*		DM	X-ray: vapor diffusion/hanging drops *3.60 Å* *3.45 Å*	Whorton and Mackinnon (2011, 2013)
Other ion channels							
GluA2 Glutamate receptor (AMPA-subtype)							
GluA2cryst: Deletion of C- and N-terminus, mutation of 3 N-glycosylaton sites, mutation of 4 residues from loop 1 by Ala, 2 point mutations (R586Q and C589A)	In complex with the competitive antagonist ZK 200775 With bound glutamate	*R. norvegicus*	Insect cells infected by baculovirus	Two point mutations (R586Q and C589A) to stabilize the tetrameric state and reduce nonspecific aggregation	C11Thio and synthetic lipids (POPC/POPE/POPG)	X-ray: vapor diffusion/hanging drops *3.60 Å*	Sobolevsky et al. (2009)
ASIC1 acid-sensing ion channel							
ΔASIC1: N- and C-terminus deletions	With bound chloride ion	*G. gallus*	Insect cells infected by baculovirus			X-ray: vapor diffusion/hanging drops *1.9 Å*	Jasti et al. (2007)

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
ASIC1mfc: amino terminal fusion with GFP and 8xHistag	Desensitized state, minimal functional channel	*G. gallus*	Insect cells infected by baculovirus		DDM	X-ray: vapor diffusion/hanging drops *3.0 Å*	Gonzales et al. (2009)
PcTx1–ΔASIC1(26-463) with His10- tag in N-terminus	In complex with psalmotoxin 1 (PcTx1)	*G. gallus*	Insect cells infected by baculovirus		DDM	X-ray: vapor diffusion/hanging drops *2.99 Å 2.80 Å*	Dawson et al. (2012); Baconguis and Gouaux (2012)
Channels: aquaporins and glyceroporins							
AQP4 aquaporin water channel							
AQP4 S180D mutant		*R. norvegicus*	Insect cells infected by baculovirus			Electron Diffraction *3.2 Å 2.80 Å*	Hiroaki et al. (2006) Tani et al. (2009)
M1 isoform full-length AQP4 with N-terminal 8xHis followed by a flag tag		*H. sapiens*	*P. pastoris*		OG	X-ray: vapor diffusion/hanging drops *1.8 Å*	Ho et al. (2009)
HsAQP5: aquaporin water channel							
HsAQP5		*H. sapiens*	*P. pastoris*		NG	X-ray: vapor diffusion/hanging drops *2.0 Å*	Horsefield et al. (2008)
Channels: Urea Transporter							
UT-B: with 8-Histag in C-terminus		*B. taurus*	Insect cells infected by baculovirus		OG	X-ray: vapor diffusion/hanging drops *2.36 Å*	Levin et al. (2012)
Channels: Amt/Rh proteins							

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
Rh C glycoprotein ammonia transporter		*H. sapiens*	HEK293 cells		OG	X-ray: vapor diffusion/hanging drops *2.10 Å*	Gruswitz et al. (2010)
Connexin gap junction							
Connexin 26 Cx26; GJB2		*H. sapiens*	Insect cells infected by baculovirus			*3.5 Å*	Maeda et al. (2009)
Intramembrane proteases							
CAAX protease ZMPSTE24. E336A mutant	In complex with C-terminus tetrapeptide from prelamin A	*H. sapiens*	Insect cells infected by baculovirus			*3.40 Å*	Quigley et al. (2013)
Antiporters							
UCP2 mitochondrial uncoupling protein 2							
Residues 14–309, C-terminal His6 tag. ^{15}N-, ^{13}C, ^{2}H labeled protein	In complex with GDP	*M. musculus*	*E. coli*	GDP	DMPC cardiolipinphytanoyl lipids and DPC	NMR	Berardi et al. (2011)
ATP-binding cassette (ABC) transporters							
P-glycoprotein							
Apo-Pgp with 3 N-linked glycosylation sites mutated and 6xHistag in C-terminus	Drug-bound: nucleotide-free inward-facing conformation competent to bind drugs	*M. musculus*	*P. pastoris*		DDM ± QZ59-RRR or QZ59-SSS	X ray: vapor diffusion/hanging drops *3.8 Å*	Aller et al. (2009)
ABCB10 mitochondrial ABC transporter							

Table 1.1 (continued)

Recombinant protein	Conformation	Source	Expression system	Stabilization strategy	Detergent used	Structural method and resolution	Reference
Apo-ABCB10: deletion of the N-terminal residues, with both N- and C-terminal His-tags	With bound ATP analogs. Open inward conformation	*H. sapiens*	Insect cells infected by bacculovirus		DDM and CHS, ± nucleotide analogs AMPPNP or AMPPCP	X-ray: vapor diffusion/hanging drops *2.85 Å*	Shintre et al. (2013)
P-type ATPases							
Calcium ATPase from sarcoplasmic reticulum							
SERCA1a-BAD: SERCA1a fused to a biotin acceptor domain	With bound $Ca^{2+}$and AMPPCP	Rabbit	*Saccharomyces cerevisiae*		C12E8 and POPC, in presence of Ca^{2+} and AMPPCP	X-ray: vapor diffusion/hanging drops *3.3 Å*	Jidenko et al. (2005)
Phospholamban							
PLN in fusion with the maltose-binding protein (MBP) Unlabeled and ^{15}N,^{13}C-labeled PLN	Unphosphorylated PLN pentamer in DPC micelles In T state	*H. sapiens*	*E. coli*		POPC/POPE lipid bilayer	Solution NMR and solid-state NMR	Oxenoid and Chou (2005); Verardi et al. (2011)

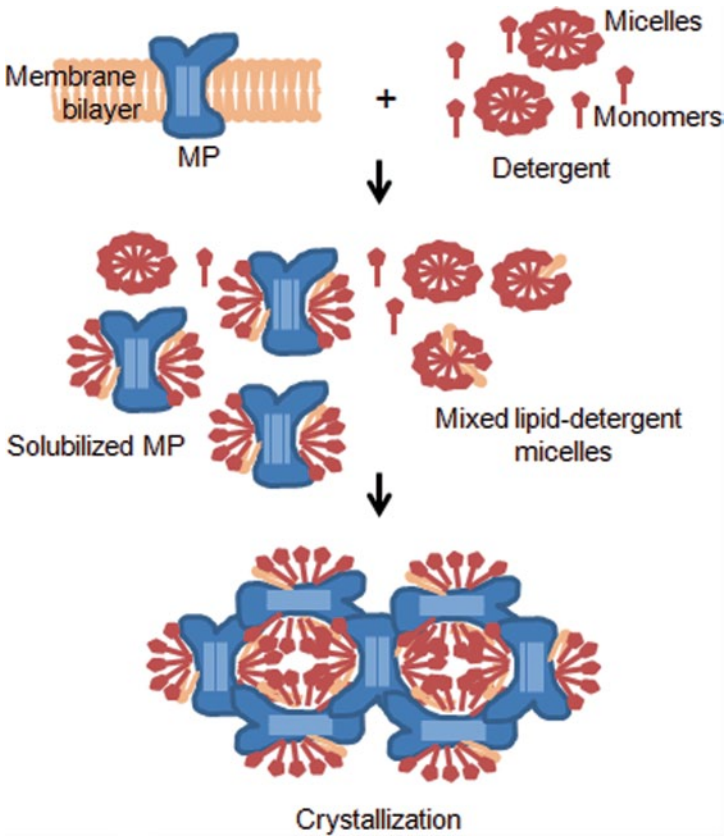

Fig. 1.1 Solubilization and crystallization of membrane proteins (*MP*s) in detergent solution

is a major obstacle to IMP structure determination. Scaffolds of the crystal lattice are found predominantly between the exposed polar surfaces of proteins, while the transmembrane parts remain buried from the detergent micelle (Fig. 1.1). Proteins with large extra-membranous domains are favored, and detergents that assemble into small micelles such as octyl-β-D-glucopyranoside (OG) or dimethyldodecylamineoxide (DDAO) are preferred in crystal trials (Prive 2007). In the past few years, structures of some recombinant "native" mammalian IMPs have been solved in OG, e.g., the aquaporin AQP4 (Ho et al. 2009), the urea transporter UTB (Levin et al. 2012), and the RhC glycoprotein ammonia transporter (Gruswitz et al. 2010); in nonyl-β-D-glucopyranoside (NG), e.g., aquaporin AQP5 (Horsefield et al. 2008); and in decylmaltoside (DM), e.g., potassium channels Kir2.2 (Tao et al. 2009) and Kir3.2 (Table 1.1; Whorton and Mackinnon 2011). Dodecyl-β-D-maltoside (DDM), a detergent that forms larger micelles, has also given rise to the structure of some recombinant "native" mammalian IMPs such as the acid-sensing ion channel 1 (Jasti et al. 2007), the potassium channel TRAAK (Brohawn et al. 2012, 2013), and the mitochondrial ABC transporter ABCB10 (Table 1.1; Shintre et al. 2013) . However,

these detergents are not good for structure determination of "native" recombinant GPCRs. Indeed, the hydrophilic regions of these proteins, which are essential to form crystal contacts, are relatively small and often occluded by large detergent micelles such as those formed by DDM, and detergents forming smaller micelles are often denaturing for GPCRs. Moreover, the dynamic character of these proteins led to conformational heterogeneity that prevents the formation of well-ordered crystals. Thus, there are several options for promoting crystal formation of such IMPs: (1) increasing the conformational homogeneity of the protein by locking it in a single conformation, (2) increasing the hydrophilic area of the protein so that large micelle detergents can be used, (3) increasing the thermostability of the protein in detergent solution, and/or (4) finding new surfactants that are able to maintain the protein conformation stable in solution and are suitable for crystallization (Fig. 1.2).

1.3.1 Increasing the Conformational Homogeneity of the Protein by Locking it in a Single Conformation

IMPs such as GPCRs, transporters, and channels are dynamic proteins that exist in several functionally distinct conformation states (active/inactive, open/closed, etc.). "Crystallogenesis typically traps the most stable low energy states, making it difficult to obtain high-resolution structures of other less stable but biologically relevant functional states" (Steyaert and Kobilka 2011). Almost all recombinant GPCR structures have been solved thanks to the binding of a high-affinity ligand, agonist, inverse agonist, or antagonist (Table 1.1). The ligand probably locks the receptor in a single conformation and stabilizes it during crystallization. However, agonist alone is not sufficient to stabilize a fully active conformation, and the structures obtained are almost from inactive conformation (Steyaert and Kobilka 2011).

Locking of a single conformation can be obtained by applying mutagenesis. Structure of rhodopsin active conformation has been solved using constitutively active mutants (Deupi et al. 2012).

Co-crystallization with monoclonal antibody fragments (Fab) that bind to a single conformational state of the protein with high affinity and specificity locks it in a specific conformation and reduces the flexibility of the loop regions, can enhance the formation of well-diffracting crystals, and gives rise to the determination of the structure of different conformations of the protein (Fig. 1.2). Co-crystallization of β2 adrenergic receptor and A_{2A} adenosine receptor with Fab fragments allowed the structure determination of an inactive conformation (Rasmussen et al. 2007; Hino et al. 2012), while active conformation of β2 adrenergic receptor in complex with a heterotrimeric G protein was solved using co-crystallization with nanobodies (Rasmussen et al. 2011). These small fragments (13 kDa) corresponding to the variable domains of the llama antibody heavy chains are characterized by outstanding properties in terms of production, stability, and recognition of epitopes buried and inaccessible to conventional monoclonal antibodies (Steyaert and Kobilka 2011; Hamers-Casterman et al. 1993; Muyldermans et al. 2001).

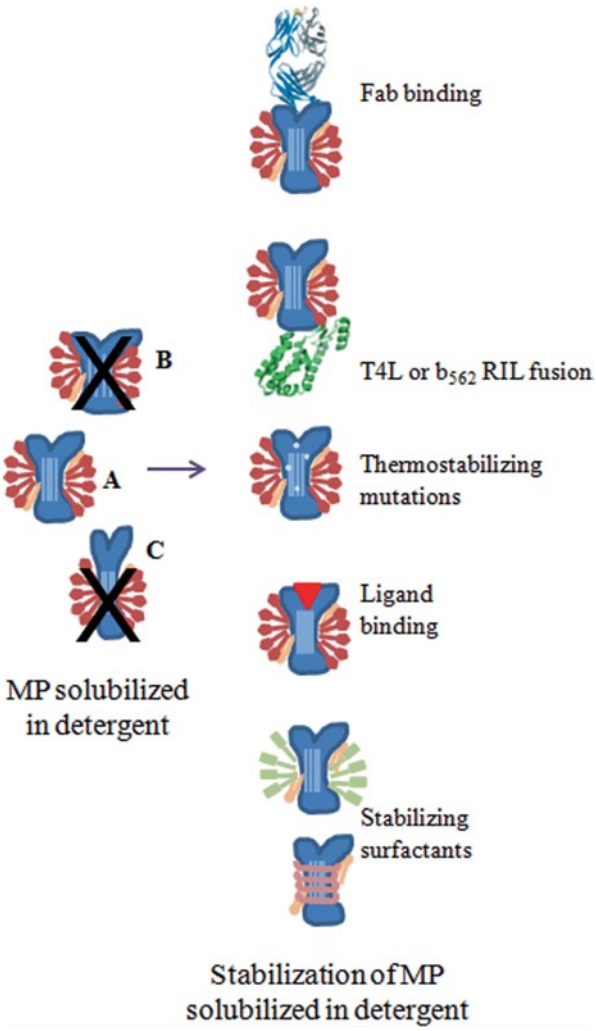

Fig. 1.2 Strategies for the stabilization of membrane proteins solubilized in detergent. *Left:* solubilization destabilizes membrane proteins (*MP*s). Presence of nonfunctional conformations in solution which prevent protein crystallization. *Right:* different stabilization strategies to preserve functional conformation in solution

1.3.2 Increasing the Hydrophilic Area of the Protein

Co-crystallization with Fab or nanobodies not only favors a given conformation but also increases the hydrophilic surface of the protein-promoting crystal formation. The probability of obtaining crystals of IMPs can also be enhanced by the fusion of stable soluble protein domain increasing the hydrophilic area. In many IMPs, regions such as N- and C-terminus and loops are highly flexible and possess large stretches of polar residues unstructured and not suitable for crystal lattice contact formation. This is the case of GPCRs. The replacement of a flexible region by a stable soluble protein domain that increases the hydrophilic area of the GPCRs has had a lot of success in determining structures of GPCRs. The replacement of

the third intracellular loop, which is highly flexible in many GPCRs, by T4L has allowed the resolution of the structure of 11 of the 19 GPCRs published to date (Table 1.1, Fig. 1.2). T4L is a very stable and highly crystallizable soluble protein. Combined with the use of LCP as the crystallization matrix, this is by far the most effective strategy to solve GPCR structure. However, a number of GPCRs were not amenable to this approach due to deleterious effects on the expression or stability of the chimeric protein. Ray Stevens and collaborators reported recently the identification and development of novel GPCR fusion proteins to facilitate GPCR crystallization (Chun et al. 2012). Of the five new fusion proteins considered in their study, thermostabilized apocytochrome b_{562} RIL showed great utility in the crystallization of several GPCRs with superior characteristics relative to T4L that has been used previously (Chun et al. 2012). The replacement of the third intracellular loop of A_{2A} adenosine receptor with apocytochrome b_{562} RIL gave rise to the highest-resolution structure of A_{2A} adenosine receptor (Liu et al. 2012, 2012a), and the replacement of the N-terminal domain of smoothened receptor with apocytochrome b_{562} RIL allowed the resolution of the first structure of this receptor (Wang et al. 2013).

1.3.3 Increasing the Thermostability of the Protein in Detergent Solution

1.3.3.1 Using Additives to Detergent

Several successful GPCR structure determination efforts have shown that the addition of cholesterol analogs is often critical for maintaining GPCR stability. Thompson and coworkers (2011) have shown that sterols such as cholesteryl hemisuccinate (CHS), which induces the formation of a bicelle-like micelle architecture when mixed with DDM detergent, substantially increases the stability of the NOP receptor ORL-1, a member of the opioid GPCR family, in a mixed micelle environment.

1.3.3.2 Using Protein Engineering

A single point mutation can increase the stability of an IMP as shown for KcsA, M13 coat protein, diacylglycerol kinase (DAGK), and bacteriorhodopsin (Bowie 2001). Rhodopsin was significantly stabilized by engineering a new disulfide bond (Standfuss et al. 2007). Thermostabilizing mutations can be additive when combined and highly stable IMPs can be engineered (Zhou and Bowie 2000; Lau et al. 1999). These mutations favor a particular state of the receptor, which enhances the probability of obtaining high-quality diffracting crystals for structure determination (see Tate 2012 for a review). Tate and coworkers developed systematic scanning mutagenesis and used a ligand-binding assay to isolate mutants highly stable in detergent solution (Fig. 1.2). This approach allowed the determination of the structure of several GPCRs carrying combination of thermostabilizing point mutations like

adrenergic receptor β1 (Warne et al. 2008, 2011, 2012; Moukhametzianov et al. 2011), adenosine receptor A_{2A} (Lebon et al. 2011), and neurotensin receptor NTS1 (White et al. 2012; Shibata et al. 2013). This strategy allowed the structure determination of GPCRs bound to low-affinity agonists or ligands which, with native protein, would lead to incomplete occupancy of the receptor and conformational heterogeneity in crystallization trials preventing the formation of well-diffracting crystals. In the case of β1 adrenergic receptor and A_{2A} adenosine receptor, the structures of thermostabilized mutants are identical to those of receptors in which the third intracellular loop was replaced by T4L, suggesting that thermostabilizing mutations predominantly affect receptor dynamics rather than the structure (Tate 2012).

For GPCRs, the use of several stabilizing strategies was necessary for the structure determination. The structures of smoothened and serotonin receptors were obtained using both apocytochrome b_{562} RIL and thermostabilizing point mutations (Wang et al. 2013; Wacker et al. 2013). The structures of chemokine receptor CXCR4 (Wu et al. 2010), dopamine D3 receptor (Chien et al. 2010), and neurotensin receptor (White et al. 2012) were obtained using T4L and thermostabilizing point mutations; and the NOP receptor structure was solved using both apocytochrome b_{562} RIL and T4L fusions (Thompson et al. 2012). In addition, often, it was necessary to truncate flexible N- and C-terminal regions of the protein.

Although these methods have been very successful and have resulted in an almost exponential growth in the number of mammalian IMPs and more particularly in GPCRs solved structures, the inherent limitations with such recombinant methods are that (1) truncations of a loop and/or of the N- and the C-terminal domains provide limited understanding of the structure and function of these regions, and (2) the locking of a conformational state using antibodies, replacement of a flexible loop by a stable soluble protein domain such as the T4L or the apocytochrome b_{562}-RIL, or the insertion of thermostabilizing mutations may affect the conformation and the structure of the protein. Thus, it is important to also develop new surfactants enhancing IMPs' stability and homogeneity, and the probability of obtaining well-diffracting crystals (Fig. 1.2).

1.3.4 New Surfactants Enhancing MP Stability and Homogeneity in Solution

To solubilize biological membranes, detergents need to be dissociating: they compete with lipid–lipid and lipid–protein interactions and disperse membrane components in the form of detergent-solubilized particles. The dissociating character of the detergent is frequently responsible for the destabilization and irreversible inactivation of IMPs after extraction (see Breyton et al. 2010 for a review). Several new classes of amphiphiles have been developed in the last decade in order to enhance IMPs' stability in solution and the probability of obtaining well-diffracting crystals such as the MNG from Samuel Gellman (Chae et al. 2010), the maltoside surfac-

tants from Wolfram Welte (Hovers et al. 2011), the fluorinated surfactants (FS) from Bernard Pucci (Breyton et al. 2004, Chap. 8 by Durand et al. in this volume), and the amphipathic polymers "amphipols" from Jean-Luc Popot (Popot et al. 2011, Chap 7 by Zoonens et al. in this volume). To date, of these different new classes of amphiphiles, only MNG has allowed obtaining crystals of sufficient quality for structure determination of mammalian IMPs.

1.3.4.1 Maltose-Neopentyl Glycol

This class of amphiphiles is built around a central quaternary carbon atom, which enables the incorporation of two hydrophilic and two lipophilic subunits, and is intended to place subtle restraints on conformational flexibility (Chae et al. 2010). In case of MNG, central quaternary carbon atom was derived from neopentyl glycol and hydrophilic groups were derived from maltose. The structures of four GPCRs were obtained using MNG. However, these four GPCRs were engineered by fusion with the soluble lysozyme domain T4L; M3 muscarinic acetylcholine receptor (Kruse et al. 2012), μ-opioid receptor (Manglik et al. 2012) and δ-opioid receptor (Granier et al. 2012), or with thermostabilizing mutations; NTS1 neurotensin receptor (White et al. 2012), and no structure of nonengineered IMP has been obtained using MNG yet. MNG is also suitable for NMR structural characterization of IMPs as shown by the recent study on the role of ligands on the equilibriums between functional states of the β2 adrenergic receptor (Kim et al. 2013).

1.3.4.2 Maltoside Surfactants

Welte and co-workers hypothesized that conformationally restricted groups in the hydrophobic part lead to mild surfactants and increase protein stability in solution. They synthesized maltoside surfactants with rigid, saturated or aromatic hydrocarbon groups as hydrophobic parts by combinations of cyclohexyl rings and aromatic rings, and maltosyl as polar head group because of its stabilizing effects (Xie and Timasheff 1997) and its compactness (Hovers et al. 2011). The use of PCC-α-M propylcyclohexyl cyclohexyl-α-D-maltoside allowed the determination of the structure of the cytochrome b_6f complex from *Chlamydomonas reinhartii* (Hovers et al. 2011). Although, this surfactant was shown to efficiently stabilize β1 adrenergic and smoothened receptors, no sufficiently well-diffracting crystal of these mammalian IMPs has been obtained using this surfactant yet.

1.3.4.3 Fluorinated Surfactants

These surfactants possess the same general structure as classical detergents, i.e., a hydrophilic head group and a hydrophobic tail, but the latter, rather than being a

hydrogenated aliphatic chain, is a fluorocarbon chain (Pavia et al. 1992, Chabaud et al. 1998). Several IMPs such as bacteriorhodopsin and cytochrome b_6f have been shown to be more stable once transferred to FSs than in the presence of detergents (Breyton et al. 2004). A similar effect was shown on the human smoothened GPCR (Nehmé et al. 2010). In contrast to detergents, FSs are not able to extract IMPs from membranes; they do not interact with lipids and do not compete with protein–lipid interaction (Rodnim et al. 2008). This character, in combination with the relative stiffness of their chains compared to detergents, is favorable for the maintenance of MP integrity and function (Talbot et al. 2009). FSs were also described as possible chemical chaperones (Rodnim et al. 2008), and were used with success for the CF production of bacterial IMPs (Park et al. 2007, 2011) and also mammalian IMPs such as the mitochondrial uncoupled protein UCP1 (Blesneac et al. 2012). Recent advances in the use of these amphiphiles for IMPs structural analysis are reviewed in Chap. 8 by Durand et al. in this volume.

1.3.4.4 Amphipathic Polymers

Amphipathic polymers (APols) have been designed to form compact and stable complexes with IMPs (Breyton et al. 2010; Popot et al. 2011). They are small (9–10 kDa), with dense hydrophobic chain distribution that ensures high affinity for the protein's transmembrane surface, high solubility in water to keep MPs soluble up to tens of grams per liter, and high flexibility to adapt to the irregularities and small radius of curvature of protein's transmembrane regions. A8-35, by far the most extensively studied APol, is composed of a relatively short polyacrylate chain (~70 acrylate residues), some of the carboxylates of which have been grafted with octylamine (~17 of them) or isopropylamine (~28 units). The ~25 acid groups that have remained free are charged in aqueous solution, which makes the polymer highly water soluble, while the octylamide moieties render it highly amphipathic (Tribet et al. 1996; Popot et al. 2011). Compare to the IMPs solubilization with detergents, the IMP/APols complex is highly stable and allows the absence of any polymer molecule free. Thus, the IMP in complex with APols behaves almost like a conventional water-soluble protein, which makes it easy to handle for functional and structural studies (Fig. 1.2). During the last decade, APols permitted major breakthroughs regarding overexpression, purification, and stabilization of IMPs, opening very exciting perspectives for structural and dynamic investigations of these proteins (Popot et al. 2011). Neutral APols (NAPols) have been shown to maintain the native folding and the activity of some GPCRs in solution (Rahmeh et al. 2012), but also to permit native folding recovery by *in vitro* refolding through strategies based on the expression of the receptors in IBs (Banères and Mouillac 2012). Recent advances in the use of APols for IMPs structural analysis are reviewed in Chap. 7 (Zoonens et al.) in this volume.

1.4 Structural Biology of IMPs

As discussed earlier, IMPs are difficult to study due to their hydrophobic properties which generate a large number of critical steps during their expression and purification for structural analysis. Despite these difficulties, the elucidation of high-resolution structures of IMPs is increasing. This progress in structural biology of IMPs results from the conjunction of biotechnological advances for expression, sample preparation (as discussed earlier), and technical improvement of structural methods for structure determination (crystallography, NMR spectroscopy, electron microscopy).

Actually, most IMP structures have been resolved by X-ray crystallography, and it is still the method of choice to elucidate large IMPs. Nevertheless, the increasing sensitivity, the development of new pulse sequences, and the production of specific labeled proteins make the use of NMR spectroscopy possible not only for structure determination but also for the characterization of the intrinsic dynamics of the protein and ligand interaction.

1.4.1 Recent Progresses in Crystallogenesis and Crystallography of IMPs

Crystallization process of IMPs is still a challenging project. Advances in protein crystallization (high-throughput screening conditions, chemical synthesis of new detergents, and chemical additives) result in a better understanding of the crystallogenesis process. *In surfo* and *in meso* phase crystallizations of IMPs are the most commonly used methods for IMPs.

1.4.1.1 In Surfo Phase Crystallization

Micellar Systems

In surfo crystallization of IMPs is the most common and easy to use strategy for the first trials for the crystallization of IMPs. In the Brookhaven data bank, the majority of deposited structures have been determined using crystals grown from detergent-solubilized proteins by traditional vapor diffusion experiments (Chayen 2005). The recent advances in the synthesis of new detergents offer a large panel suitable for the purification and crystallization of IMPs (Kang et al. 2013). The choice of the detergent is crucial and depends on many different parameters including extraction and solubility efficiency, protein stability, and retention of function. Accounting the crystal structures of IMPs listed on the webpage http://blanco.biomol.uci.edu/mpstruc/, alkyl maltopyranosides or glucopyranosides are the most successful detergents followed by amine oxides and polyoxyethylene glycol (Parker and Newstead 2012). Actual screens dedicated to the crystallization of

IMPs are commercially available and are very useful for the first crystallization trials. The stability of the protein during the crystallization process often needs additional lipids. Lipids have become more and more important during the crystallization process and can play a role as molecular chaperone for IMPs. Cholesterol is necessary for the crystallization of GPCRs, and it was shown that cholesterol molecules formed the interface between the protein molecules in the physiological state (Cherezov et al. 2007). However, not all IMPs can be maintained in native conformation when solubilized with conventional detergents. Recently, new synthesis of detergents has been proposed to overcome the stability problem occurring during extraction of IMPs and to favor their crystallization by limiting the size of the micelles surrounding the protein. These new detergents, discussed before, are based on DDM. By adding two hydrophilic and two lipophilic subunits linked by a central quaternary carbon, MNG is a better detergent compared to DDM for extraction, stabilization, and crystallization of IMPs (Chae et al. 2010). Another detergent called facial or tandem facial amphiphile has been developed by (Zhang et al. 2007) to favor the stability of solubilized IMP by forming small micelles compared to DDM and are found to be more suitable for the crystallization and NMR spectrometry of IMPs.

Nonmicellar Systems

Once IMPs are extracted from their natural environment, they are vulnerable. Detergents are a relatively poor substitute for the bilayer and IMPs are often unstable outside the membrane. Slight perturbation of MP structure may lead to denaturation and aggregation after detergent extraction. Furthermore, obtaining crystals means screening various biochemical and/or biophysical conditions (pH, ionic strength, additives, temperature, etc.) which may alter the tiny equilibrium for the micelle/protein complex. One way to stabilize IMPs is to reconstitute them in mimicking phospholipid bilayer membranes like bicelles or nanodiscs.

The bicelles are lipid bilayers limited in size. The size is determined by the ratio between a long chain of phospholipids, generally phosphatidyl choline (like dimyristoyl phosphatidylcholine (DMPC) or ditridecanoyl phosphatidylcholine (DTPC), and short lipids like cholate or short phospholipids like dihexanoyl-phosphatidylcholine (DHPC) that form the rim. Bicelle is an attractive lipidic medium for mimicking phospholipid membranes. In the case of bacteriorhodopsin (Faham and Bowie 2002), clear density for a CHAPSO molecule inserted between protein subunits is seen within the layers, indicating that an important interaction between lipids and protein has been preserved within the bicelle by the cholesterol-like detergent. Thus, this method has the advantage to be a bilayer-based method, to preserve integrity of the protein, and the ability to diffuse in three dimensions to form a three-dimensional (3D) lattice. Moreover, the ability to grow crystals at room temperature (below the phase transition temperature) significantly expands the applicability of bicelle crystallization (Ujwal and Bowie 2011). Proteins in bicelle can be handled like proteins in detergent. Bicelle crystallization trials can be performed

like the standard detergent-based protocol including robotics and all commercially available screens.

Nanodiscs have been extensively used for solubilizing IMPs and present similar advantages as bicelles in terms of size and stability (Bayburt and Sligar 2010). The nanodisc is a noncovalent assembly of phospholipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC; dipalmitoylphosphatidylcholine, DPPC, and dimyristoylphosphatidylcholine, DMPC) and a genetically engineered "membrane scaffold protein" (MSP) based on the sequence of human serum apolipoprotein AI (Bayburt and Sligar 2010). The molecular ratio of phospholipids to MSP is crucial for right self-assembling particle formation. The phospholipid associates as a bilayer domain while two molecules of MSP wrap around the edges of the discoidal structure in a belt-like configuration, one MSP covering the hydrophobic alkyl chains of each leaflet. The MSPs were engineered into the synthetic gene optimized for expression in *E. coli* and include various affinity tags (6His, FLAG, Cys, etc.). The size of nanodiscs can be adjusted depending on the length of MSP (Hagn et al. 2013). Nanodiscs are self-formed from a mixture of detergent/phospholipid micelles and MSP upon removal of the detergent (Ritchie et al. 2009). Nanodiscs are then an ideal model membrane system with defined size and phospholipid composition. Despite their properties, the use of nanodiscs as mimicking membrane for crystallization is still quite limited and efforts must be accomplished to increase the packing contact necessary for crystallization. The small isotropic nanodiscs are more useful to study specific lipids/proteins and proteins/proteins interactions for a better understanding of MP function (El Moustaine et al. 2012).

1.4.1.2 *In Meso* Phase Crystallization: The LCP Method

Crystallization of IMPs *in meso* phase has emerged as the most powerful method, in particular for GPCRs structure determination. This method for crystallization of IMPs was first originally described by Landau and Rosenbusch (1996) using bacteriorhodopsin. Actually, the advances of a robotic system for the crystallization of IMPs *in meso* phase result in more than two thirds of GPCR's crystallographic structures (Cherezov 2004). The *meso* phase is a bicontinuous lipidic phase formed spontaneously by mixing monoacylglycerols (MAGs) and water at a given ratio (Caffrey and Cherezov 2009a). The cubic phase can be doped by essential lipids like cholesterol or phospholipids for the stabilization of the protein of interest. LCP is composed of highly curved lipid bilayers and is connected by a water channel. The MP solubilized in detergent is added to the LCP, the lipids present at high concentration will replace the detergent molecules, and thus the protein will be reconstituted into the lipid bilayer. IMPs are able to diffuse freely in lipids and therefore make contact with each other for the nucleation and crystallization process. LCPs are viscous and not easy to handle for crystallization trials. But during the past few years, many tools and instruments have become available, making LCP for IMP crystallization a routine application (Caffrey and Cherezov 2009). Moreover,

the development of methods for measuring thermal stability and diffusion of MPs embedded in LCP seek to identify precisely the best condition for crystallization.

1.4.2 Data Collection and Structure Determination from MP Crystals

Data collection of IMP crystals is still very challenging. In the *in surfo* phase, the crystals usually have a high solvent content owing to the detergent micelle, which covers the hydrophobic part of the protein. Therefore, the crystals of IMPs are often fragile, difficult to handle, and suffer from anisotropic X-ray scattering and radiation sensitivity. In a LCP, the crystals generally contain less solvent, which makes them less radiation sensitive, but the crystals are often much smaller. In addition, crystal quality can vary considerably, even between crystals from the same drop. This means that the complete X-ray data of an IMP crystal require screening a large number of crystals at the synchrotron. The presence of automatic sample changers at synchrotron beamlines has helped to solve this problem, enabling many crystals to be screened quickly and efficiently (Blow 2008). Moreover, the microfocus beamlines at the synchrotron (Bowler et al. 2010) make data collection possible from the undersized crystals or on focused patches of the best-ordered regions of larger crystals. Focused X-ray beams with low background scatter and beam sizes of less than 10 μm remarkably improve the resolution and the data statistics from small crystals. The disadvantage of these microfocused beamlines is to increase the radiation damages of crystals; however, this can be overcome by merging data from several crystals.

Phase determination of IMP crystals can also be very challenging. However, with the increasing number of solved structures, molecular replacement (MR) has nonetheless become the most successful method to obtain phase information (Bill et al. 2011). Actually, most of GPCR structures recently published have been solved using MR. Co-crystallization of the protein of interest with a protein domain of well-known structure (e.g., T4L fused in the intracellular loop of GPCRs) can also be used as a search model to obtain phase information. However, if the structural fold is not known, experimental phase determination is required. Classical crystal-phase determination methods used for soluble proteins can be applied such as selenomethionine labeled protein or heavy metal derivatives.

Serial femtosecond crystallography (SFX) using X-ray free-electron laser (XFEL) radiation is an emerging method for 3-D structure determination using crystals ranging from a few micrometers to a few hundred nanometers in size. This method relies on X-ray pulses that are sufficiently intense to produce high-quality diffraction but short enough to prevent substantial radiation damage (Chapman et al. 2011). X-ray pulses of only 70-fs duration terminate before any chemical damage processes have time to occur, leaving primarily ionization and X-ray-induced thermal motion as the main sources of radiation damage (Holton 2009). SFX therefore promises to break the correlation between sample size, damage, and resolution in structural biology.

1.4.3 Recent Progresses in NMR Spectroscopy of IMPs

According to the structure of IMPs deposited in PDB and by comparing the two lists of X-ray structures (http://blanco.biomol.uci.edu/mpstruc/) and NMR structures (http://www.drorlist.com/nmr/MPNMR.html), the larger part of IMP structures have been solved by X-ray crystallography; nevertheless, recent advances in NMR spectroscopy, both in liquid and solid states, have made comprehensive studies of larger IMPs more accessible. NMR structures of IMPs contribute to 10–15 % of the overall structures (Nietlispach and Gautier 2011). This contribution is due to the recent advances that appeared not only in NMR spectroscopy but also in biotechnology for labeling and stabilizing IMPs in large amount for structural biology (as mentioned previously).

One advantage of NMR spectroscopy over the X-ray structure determination is the measurement of intrinsic dynamics of the protein in native-like conditions (Warschawski et al. 2011). NMR spectroscopy can be used with IMPs in several environments: in isotropic environments, in bicelles, or in anisotropic environments such as proteins embedded in phospholipid membranes in physiological conditions (Warschawski et al. 2011). According to the sample preparation, NMR in solution or ssNMR spectroscopy will be used. More than 90 % of the IMPs structures determined by NMR have been elucidated in solution. But the recent progress in ssNMR offers great potentiality, as illustrated in studies of large IMPs like the phospholamban (Traaseth et al. 2009; Verardi et al. 2011), the influenza proton channel (Cady et al. 2009; Cady et al. 2010; Sharma et al. 2010), the *Yersinia enterocolitica* adhesin A (Shahid et al. 2012), and the structure of a GPCR (Park et al. 2012). However, structure determination by NMR spectroscopy of IMPs remains a challenging project. The major limitations of NMR structure determination still persist because of sample preparation, in particular preparation of large amount of isotope-labeled proteins, and the choice of the mimicking environment to keep the proteins stable during the long-time NMR experiments.

1.4.3.1 Strategy for Isotopic Labeling of IMPs

Most of IMP structures determined by NMR spectroscopy have been overexpressed in *E. coli*. This prokaryotic expression system is the most appropriate expression system for fully ^{15}N-, ^{13}C-, and ^{2}H-labeled IMPs. As discussed previously, a large variety of expression plasmids and strains are available for heterologous expression of IMPs for this purpose (Freigassner et al. 2009; Kainosho et al. 2006; Schlegel et al. 2012; Vaiphei et al. 2011). Yeast and mammalian cell expression systems are also being developed as alternate sources of isotope-labeled proteins (Egorova-Zachernyuk et al. 2011; Fan et al. 2011; Sarramegna et al. 2003). CF systems based on *E. coli,* wheat germ, or insect cell extracts (see Chap. 2 from Proverbio et al. in this volume) are an alternative for expression of uniformly ^{15}N, ^{13}C-labeled IMPs as

well as perdeuteration (Etezady-Esfarjani et al. 2007; Sobhanifar et al. 2010a). This strategy of expression overcomes the limitations occurred *in vivo* expression systems, and is very useful for the selective labeling of amino acids. CF system expression was recently used in combination with a sequence-optimized combinatorial dual-labeling approach to achieve rapid backbone assignment for a two-α-helical, a two-transmembrane, and a four-transmembrane histidine kinase receptors (Hefke et al. 2011; Maslennikov et al. 2010).

Additionally, it is noteworthy that fluorine labeling of IMPs could be very useful in NMR spectroscopy, for example, to define conformational state in IMPs. A covalent modification of cysteines with 19F-labeled compound was used to describe conformational changes in β2-adrenergic receptor (Liu et al. 2012a). Moreover, incorporation of unnatural fluorinated amino acid in the DAGK was used to evaluate the dynamics of this MP in *N*-dodecylphosphocholine (DPC) micelles and in natural *E. coli* membrane (Shi et al. 2012; Shi et al. 2011).

1.4.3.2 Membrane-Mimicking Environment for IMP Structure Determination by NMR

For Solution NMR Spectroscopy

One of the challenges for solution NMR spectroscopy of IMPs is the identification of conditions that can mimic the native lipid bilayer environment while maintaining the sample in a stable, folded state with a total complex size of ~100 kDa or less (Kim et al. 2009; Sanders and Sonnichsen 2006). Micelle-forming detergent is the most common way to solubilize IMPs for NMR structural studies. A large panel of detergent molecules is actually available. Screening condition is required for finding a suitable detergent and right biochemical conditions to ensure the solubilization of IMPs in native conformation. Detergents that have been used successfully for solution-state NMR spectroscopy of IMPs include *N,N*-dimethyldodecylamine *N*-oxide (LDAO), sodium dodecyl sulfate (SDS), CYFOS-7, β-OG, and DPC (see http://www.drorlist.com/nmr/MPNMR.html). For an appropriate balance between solubilization and stabilization, mix-micelle has been used. For example, NMR spectra of the KvAP voltage-dependent K+ channel were found to be optimal in the mixture composed of a ratio of 2:1 DPC/LDAO, in which DPC alone yielded exchange-broadened NMR spectra, while LDAO yielded sharp spectra but short lifetimes (Shenkarev et al. 2010). In some cases, detergent micelle can be doped by addition of natural phospholipids as was required for UCP2 (Berardi et al. 2011).

Bicelles have emerged as a common medium for use in NMR studies of IMPs. Many recent structures have been solved by using isotropic bicelles composed of DMPC phospholipids and small acyl chain phosphatidyl choline DHPC (Bocharov et al. 2008; Bocharov et al. 2007; Mineev et al. 2010; Sharma et al. 2010; Shenkarev et al. 2013; Unnerståle et al. 2011). Bicelles provide medium that allows both solid state and solution NMR. Playing with the *q* value, corresponding to the ratio be-

tween the concentration of DHPC (short acyl chain) and DMPC (long acyl chain), we can increase the size of bicelles which become no more isotropic and can then be used for either ssNMR or crystallography (De Angelis et al. 2006; De Angelis and Opella 2007; Rasmussen et al. 2007).

Other non-detergent molecules have been developed for solubilization of IMPs and are very useful for NMR spectroscopy (Dahmane et al. 2009; Popot et al. 2011). As mentioned before, APols are short amphipathic polymers that can substitute for detergents to keep IMPs water soluble and stabilized. IMPs solubilized with APols have been used in NMR spectroscopy to define the conformation of the leukotriene B4 bound to its receptor (Catoire et al. 2010).

Nanodiscs are new membrane mimetic media, which are closest artificial media to the natural phospholipidic membrane with size suitable for solution NMR studies (Ritchie et al. 2009). The major advantage of nanodiscs is the absence of any detergents, which are known to affect the stability of the IMP by interacting with the extra-membrane regions. Originally developed for the solubilization of functionally active IMPs, they have since been used for ssNMR (Kijac et al. 2007), and more recently, solution NMR applications (Raschle et al. 2009). In a recent study, design of novel nanodiscs with more limited size shows 30 % reduced apparent correlation time compared to the classical one. Using this property, NMR structures of the IMP outer membrane protein-X (OMP-X) have been characterized and compared with those obtained in several micelle compositions (Hagn et al. 2013).

For ssNMR Spectroscopy

In ssNMR, the absence of a direct correlation between molecular size and sample line widths allows the use of bigger membrane media such as large bicelles or lipid bilayers, which provide a more native environment for functional proteins. The homogeneity of the sample leads to improved line widths and therefore spectral resolution. Recently, the ssNMR structure of the CXCR1 GPCR fully ^{15}N, ^{13}C-labeled was determined after reconstitution in phospholipid liposomes (Park et al. 2012). Biological membranes consist of highly complex lipid mixtures of varying compositions which can adaptively adjust to changes in the physical properties of the membrane.

1.4.3.3 Recent Developments in NMR

In Solution NMR

Transverse relaxation optimized spectroscopy (TROSY)-based experiments have expanded the applicability of 3-D structures of large protein complexes including MPs solubilized in mimicking-membrane environment (Konrat et al. 1999; Salzmann et al. 1999; Yang and Kay 1999). 3-D-TROSY version of the HNCA, HN(CO)CA, HN(CA)CB, HNCO, HN(CA)CO, etc., are used for the complete

resonance assignment. To avoid unfavorable relaxation properties of protons in large proteins, all the non-exchangeable carbon-bound protons are replaced by deuterium. Sparse proton density in perdeuterated amide HN back-exchange proteins leads to few long-range nOes. To overcome this problem, a powerful method to reintroduce a perdeuterated sample is to biosynthetically incorporate protonated methyl groups of leucine, valine, and isoleucine by growing samples in minimal media with selectively labeled α-ketoisovalerate and α-ketobutyrate (Goto et al. 1999). This method was successfully applied for the structural determination of numerous IMPs from the β-barrel family at first with OMP-X in DHPC (Hilty et al. 2002), Kp OMP-A (Renault et al. 2009), and VDAC (Hiller et al. 2008). Other methyl protons can be targeted, such as those of alanine or methionine, by adding protonated amino acid into the culture media. This specific labeling approach is very useful for NMR structure determination of large MPs but also provides large application to study the intrinsic dynamics of very large proteins like the 670-kDa 20S core-particle proteasome (Religa et al. 2010; Ruschak et al. 2010), the DDM-solubilized KcsA channel (Imai et al. 2010), and β2 adrenergic receptor (Bokoch et al. 2010).

Structure determinations by NMR spectroscopy are issued from the chemical shifts and nOes observed in 2-D, 3-D, or 4-D spectra. Distance restraints obtained by measuring nOes are often not enough to define precisely a structure of protein. Long-range restraints are necessary to obtain a more accurate NMR structure. Residual dipolar coupling (RDC) and paramagnetic relaxation enhancement (PRES) have been developed and nicely detailed in reviews (Kim et al. 2009; Qureshi and Goto 2012) and in Chap. 12 by Catoire et al. in this volume.

In ssNMR

ssNMR spectroscopy becomes a more and more attractive method for structure determination of IMPs in their native environment. The recent advances described (see for reviews Baldus 2006 and Renault et al. 2010) offer a large panel of recent developments and applications for structural and dynamic information of the large MP complex in phospholipid membrane. Moreover, technical improvements for ssNMR spectroscopy: design of the new probe heads to give access to ultrahigh-speed ssNMR and dynamic nuclear polarization (DNP) will promote ssNMR for structural biological studies of IMPs in native membranes.

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