

Section 1

Expression and Purification of Membrane Proteins

CHAPTER 1

Refolding of G-Protein-Coupled Receptors

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1 Introduction

G-protein-coupled receptors (GPCRs) are transmembrane receptors that are involved in the recognition and transduction of messages as diverse as light, Ca^{2+} ions, odorants, small molecules (including amino-acids, nucleotides, peptides) and proteins.^{1,2} Although different classes of receptors have been described,³ they all share a common structural motif composed of seven α -helices spanning the plasma membrane.

Although significant progress has been made within the last few years in dissecting GPCR-mediated signal transduction pathways, understanding the mechanisms underlying ligand recognition and signal transduction across the membrane has been hampered by the lack of information at the molecular level. This is largely due to the low abundance of most GPCRs in cellular membranes. Furthermore, few expression systems have proven satisfactory for producing these receptors in a functional state and sufficient yields.^{4–6} Structural information on the GPCR family is therefore very sparse, with the exception of rhodopsin for which X-ray⁷ and electron⁸ diffraction data have been obtained.

Recombinant expression has been one of the major bottlenecks in structural biology of GPCRs.⁹ One of the most widely used expression systems for structural biology is *Escherichia coli*. However, in general, bacterial expression has been hampered by the relatively low yields of GPCRs owing to the toxic effects caused by these 7TM receptors when inserted into the bacterial membrane.⁶ To circumvent this toxicity problem, GPCRs can be directed to bacterial inclusion bodies. This leads to high expression levels of the receptor (in the range of 10–50 mg of protein per liter of bacterial culture). However, the highly expressed recombinant receptors are inactive and require refolding into a functional form. This explains why intensive work has been developed during the last years in analyzing the refolding of GPCRs

and in devising efficient strategies for refolding receptors solubilized under denaturing conditions.

Understanding the basic principles of membrane protein folding is also of great interest in a fundamental perspective. Many studies have been dedicated in the past years to understand the trafficking of GPCRs.¹⁰ The quality control process in the endoplasmic reticulum involves a variety of mechanisms. These mechanisms ensure that only correctly folded proteins are directed to the plasma membrane. Despite this stringent quality control mechanism, gain- or loss-of-function mutations affecting protein folding in the endoplasmic reticulum, which have been described, can have profound effects on the health of an organism. Understanding the molecular mechanisms of protein folding could therefore help in correcting the structural abnormalities associated with misfolded receptors.

There are essentially two main types of membrane-spanning structures: transmembrane α -helices and β -barrels. The latter appear to be limited to outer membrane proteins. The present work will focus on the refolding of α -helical membrane proteins. This is the most general case and the most interesting from a pharmacological point of view. It applies, in particular, to the GPCR family.

Investigating the *in vitro* refolding of membrane proteins is a difficult task, in particular, due to the hydrophobic nature of integral membrane proteins. Indeed, to work with isolated membrane proteins, one has to manipulate refolding solvents, generally composed of detergents or detergent/lipid mixtures, which poorly mimic the natural membranes. Nevertheless, biophysical studies on model systems have begun to provide a sound physical basis for membrane protein folding.¹¹

2 Refolding of Membrane Proteins

As stated above, one of the greatest problems in setting up conditions for studying a membrane protein *in vitro* arises from the relative instability of these proteins in detergent solutions. This problem, associated with the low abundance of most GPCRs in cellular membranes, explains the limited number of examples of refolding studies with GPCRs. Most of the biophysical studies on α -helical membrane protein folding have been carried out with bacteriorhodopsin as a model system, since it is a membrane protein that can be purified in high yields and is relatively stable in solution.¹² Moreover, high-resolution structures of bacteriorhodopsin are available^{13,14} that help to understand the folding of this protein on a molecular basis. Bacteriorhodopsin functions as a light-driven proton pump in the purple membrane of the archaeobacterium *Halobacterium salinarium*.¹⁵ As is the case with GPCRs, it possesses seven transmembrane α -helical segments. Although it may not be fully representative of GPCRs at the folding level,¹⁶ it is nevertheless a good model system to gain a better understanding of receptor folding. Some general rules for α -helical membrane protein folding have been inferred from folding/unfolding studies with bacteriorhodopsin. In particular, a two-stage model has been proposed for the folding of these proteins that decomposes this process.^{17,18} First, individually stable transmembrane helices form and then these helices pack to form a functional protein (Figure 1).

The first step in the two-stage model thus involves the formation of the helical segments, and transmembrane helices display some characteristic features.¹⁹ In general, they are largely hydrophobic sequences that include a limited number of polar or

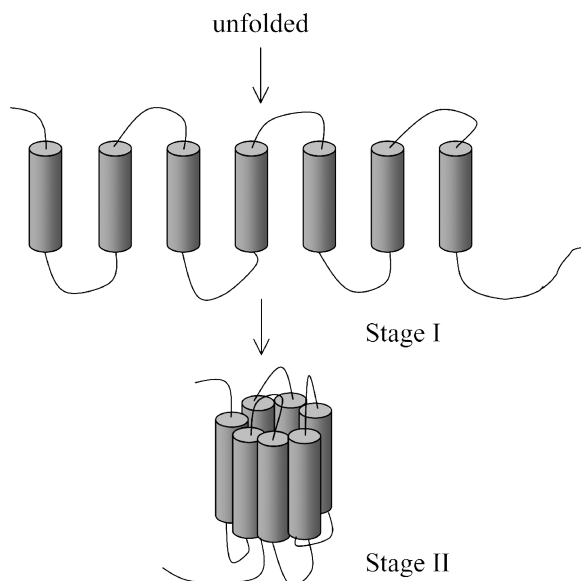


Figure 1 Schematic representation of the two-stage model for membrane protein folding (18). The transmembrane helices are considered as individually folding units. In the first stage of the folding, these domains form separately due to specific hydrophobic effects and hydrogen bonding. These helical domains then establish intramolecular contacts that lead to the native fold

potentially charged groups. In contrast to what is observed in globular proteins, prolines and glycines are also often found in membrane helices. The bending of a helical segment induced by a proline residue could be an important feature for membrane protein function. This is, for example, the case for the GPCR rhodopsin, where kink-inducing proline residues are found at key positions of the three-dimensional structure.⁷ Individual transmembrane helical structures are stable essentially because of hydrophobic effects and hydrogen bonds that are strong in the low dielectric environment of the membrane. Many helices found in membrane proteins are likely to be considered as stable folding units.²⁰ In agreement with this view, individual helical segments or fragments of bacteriorhodopsin or GPCRs, containing only a reduced number of helices, can reach a stable fold in a membrane mimicking environment (see below).

If individual helices are formed in response to main-chain hydrogen bonding and hydrophobic effects, other interactions are likely to be involved in their assembly in the second stage of refolding. An important factor is certainly the way that the helices fit together, guided by Van der Waals interactions and side-chain rotamers.^{18,19} Another factor that is likely to influence the assembly of the transmembrane helices is the lipid environment. Besides its general role as a solvent, the lipid may also stabilize membrane proteins through specific interactions. Indeed, there are many examples of specific associations of individual lipids or of classes of lipids with membrane proteins.^{21–23} The interhelical loops can also have a role in the formation of helix/helix contacts, although the fact that proteins can in some cases assemble from fragments to form functional species suggests that the constraint induced by the loops may not be essential for

folding. The loops could nevertheless promote folding events that bring transmembrane helices together. In agreement with this view, in the case of bacteriorhodopsin, the specific conformation of all the protein loops, except the DE loop, contributes to protein stability and is required for the correct folding and function of the protein.²⁴

In closing this part, although it may not fully represent the folding process of GPCRs,¹⁶ the two-stage model is certainly a good working basis for a better understanding of the folding of membrane proteins. A three stage model has also been proposed that, in addition to the two first steps, introduces a third step corresponding to ligand binding, folding of extramembranous loops, insertion of peripheral domains and formation of quaternary structures.²⁵

3 *In Vitro* Protein Refolding

Inclusion body production is a recurrent theme in recombinant protein technology. Refolding of inclusion bodies consists first in solubilizing the protein under denaturing conditions and then refolding is initiated by the removal of the denaturing agent. This can be done by dialysis or dilution. Protein folding has also been achieved by binding the protein to a chromatographic resin in the unfolded state and subsequent washing with an appropriate buffer that contains no denaturant. Affinity resins, such as Ni-NTA Sepharose^{26,27} or heparin Sepharose²⁸ have been used for poly-histidine-tagged or poly-arginine-tagged proteins, respectively. Compared to folding by dialysis or rapid dilution, this method has the main advantage of preventing aggregation due to intermolecular interaction of partly folded protein species. However, an interference of the chromatographic support with the folding protein molecule may be detrimental, especially in the case of the highly hydrophobic membrane proteins, possibly causing precipitation of the protein on the matrix.

The efficiency of refolding depends on the competition between protein refolding and aggregation. One of the main difficulties in refolding membrane proteins is thus to find conditions that favor refolding over aggregation. A delicate balance must be reached between too harsh or too mild environments. Protein folding screens for identification of optimal folding conditions have been developed during the past years to screen different factors that may influence globular protein refolding.^{27–32} In a general manner, this consists in screening multiple conditions, in which different parameters (additives, pH, salt and protein concentration) are altered. Such folding screens can be adapted to integral membrane proteins, keeping in mind that one of the most crucial parameters to test will be the nature of the detergent and/or detergent/lipid mixture. This is indeed likely to be the factor with the most dramatic effect on membrane protein refolding efficiency.

4 GPCR *In Vitro* Refolding

4.1 Resolubilization from Inclusion Bodies

In most cases, refolding of GPCRs has been carried out with material recovered from bacterial inclusion bodies.^{27,33–35} As far as expression in *E. coli* is considered, it seems that there is no general strategy to be used for the efficient accumulation of

GPCRs in inclusion bodies, even if some rules have been inferred from studies with different receptors.³⁶ In some cases, the receptor simply fused to a T7 tag was efficiently expressed in *E. coli*. This is the case, for example, for the leukotriene B₄ receptor BLT1²⁷ or, more recently, for the V2 vasopressin receptor.³⁷ However, it must be noted that in the case of BLT1, protein expression levels dramatically vary from one clone to another. In other cases, fusion of the receptor to a protein partner was absolutely required for its expression. Different partners, such as glutathione S-transferase (GST)^{33,36} or ketosteroid isomerase (KSI)³⁴ have been used. It must be emphasized again that no system allowing the expression of “all” GPCRs in inclusion bodies has been described so far. For example, no expression was observed with the 5-HT_{4a} receptor fused to GST, whereas this receptor was efficiently produced when fused to KSI.³⁴ Similarly, in our hands, among all the receptors we tested, KSI fusions gave inclusion bodies only with the 5-HT_{4a} receptor. The best approach may therefore be to test different fusion partners and then quantify the expression levels.

Before starting the refolding, the inclusion body material has to be solubilized. Globular proteins in inclusion bodies can be solubilized in the presence of high concentrations of chaotropic agents, such as guanidinium hydrochloride or urea. In contrast, aggregated membrane proteins require detergents (or organic solvents) for efficient solubilization, due to the predominance of hydrophobic effects in the aggregated material. Usually, SDS is used as a strong denaturing detergent. However, it must be kept in mind that, in SDS, helical membrane proteins, such as bacteriorhodopsin or GPCRs are not totally unfolded, but they can retain a significant amount of secondary structure. Indeed, sequence regions that, in the folded structure, form transmembrane helices tend to locally adopt an α -helical structure even in SDS. Bacteriorhodopsin, for example, is about 40–45% helical in SDS.^{38–40} The 5-HT_{4a} receptor is also 30–35% helical in SDS-containing buffers (Banères, unpublished data). The SDS-solubilized starting point for refolding is thus not to be considered as a fully unfolded state but rather as a partially folded state, as far as the secondary structure is concerned.

4.2 Refolding

As stated above, “unfolded” membrane proteins are first solubilized in harsh detergents, such as SDS or lauroylsarcosine. Refolding will then consist in replacing this denaturing detergent by a detergent that will stabilize the three-dimensional fold of the membrane protein. Under these conditions, based on the two-stage model, regions that have a propensity to fold will do so and then interactions between protein segments will appear. Those can be intramolecular, leading to refolding or intermolecular, leading to aggregation. One of the main challenges in refolding membrane proteins is therefore to find conditions, in particular, the nature of the detergent environment, which will favor the intramolecular over intermolecular contacts, and therefore refolding over aggregation. This implies finding the right balance between harsh and mild environments. For GPCRs, however, in the absence of extensive examples of successful receptor refolding, it is difficult to infer a general rule for the lipid and detergent requirements for maximal refolding efficiency.

Two different refolding studies have been reported so far for GPCRs. In the first case, the refolding involved, as described for bacteriorhodopsin, peptides encompassing

one or several transmembrane domains obtained either by peptide synthesis, restricted protease digestion of receptors or bacterial expression in inclusion bodies.^{41,42} In the second case, the intact receptor was produced in bacterial inclusion bodies and then refolded *in vitro*.^{27,34,43}

4.3 Refolding of GPCR Fragments

We will not consider here the refolding of the extracellular ectodomains of GPCRs that has been described for several receptors,^{29,44,45} since these domains behave as typical globular proteins. We will focus here only on the transmembrane regions of GPCRs. Several papers reporting the refolding of fragments containing some of the transmembrane helices of GPCRs have been published so far.^{41,42} These fragments range from a single transmembrane domain to several transmembrane helices. As predicted by the two-stage model for membrane protein refolding, these fragments form folded domains when transferred from denaturing conditions to a milder environment. This suggests that GPCR transmembrane helices can also be considered as individual folding units. Besides their interest for a better understanding of the molecular processes involved in receptor folding, these studies indicate that the study of receptor fragments could be an alternative to the analysis of the structural properties of GPCRs. We will provide here two recent examples to illustrate this aspect of GPCR refolding.

The first is that of the μ -opioid receptor for which the refolding of an 80-residue fragment has recently been described.⁴¹ The fragment, produced in *E. coli* inclusion bodies as a fusion with GST, encompassed the second and third transmembrane segments as well as the first extracellular loop of the receptor. In this case, simply by exchanging the harsh detergent lauroylsarcosine, used for inclusion body solubilization, to milder lysophosphatidylcholine micelles, a significant amount of secondary structure was recovered. Under these conditions, the receptor fragment adopted approximately 50% α -helical structure, consistent with the assumption of an α -helical structure in the two membrane-spanning regions and a non-helical structure in the loop region connecting the second and the third transmembrane domains.

The production of the seven transmembrane domains of the adenosine receptor as individual synthetic peptides has also been recently reported.⁴² In this case, circular dichroism (CD) spectra indicated that each of the seven peptides form stable, independent α -helical structures in both detergent micelles and lipid vesicles. In particular, the peptides corresponding to the third, fourth, sixth and seventh transmembrane domains exhibit high-helical structure content, close to the predicted maximum for their transmembrane segments. The peptide corresponding to the first transmembrane domain also adopts a relatively high content of α -helical structure. Interestingly, the measured helical content of some transmembrane domains does not directly correlate with the predicted helicity based on amino acid sequence. This points out that, while hydrophobic interactions can be a major determinant for folding of transmembrane peptides, other factors, such as helix-helix interactions may play significant roles for specific transmembrane domains. Such an observation suggests that, although the transmembrane peptides may essentially be considered independent folding units, interactions between the transmembrane domains could be required to some degree for proper insertion and folding of some helical domains.

4.4 Refolding of Intact GPCRs

In vitro refolding of some intact receptors has also been reported during the last 10 years. The first bacterially expressed GPCR to be successfully refolded *in vitro* has been the OR5 olfactory receptor.⁴³ Subsequently, the refolding of two other GPCRs, namely the leukotriene B₄ receptor BLT1²⁷ and the serotonin receptor 5-HT4a,³⁴ has been described. For all three receptors, the same general approach was used (see Table 1), and the reader is referred to Ref. 27, 34 and 43 for a step-by-step procedure. They all were produced in *E. coli* IB, thus allowing large protein quantities to be recovered (in the 10 mg range). The receptors were then solubilized with a harsh detergent. In the case of OR5, the overexpressed protein was solubilized in the strong, negatively charged detergent, lauroylsarcosine, whereas BLT1 and the 5-HT4a receptors were solubilized in the presence of both urea and SDS. The receptors were then refolded by solvent exchange. In all cases, solvent exchange was carried out with the receptor immobilized on a solid Ni-NTA matrix. This could indicate that for integral membrane proteins, preventing non-specific protein/protein interactions that favor aggregation, could be a crucial point to get acceptable refolding yields. At least in our hands, the matrix-assisted procedure systematically yielded significantly higher refolding yields compared to dialysis or dilution (Banères, unpublished data).

Lacking a detailed theoretical understanding of the various factors affecting GPCR refolding yields and tendency to aggregate, the strategy used in all the reported cases of receptor refolding was to vary different parameters in a systematic way and quantify the refolding yield. One of the most crucial parameters was, as expected, the composition of detergent/lipid micelles. The OR5 was first reconstituted in the non-denaturing detergent digitonin. In this detergent, the receptor was able to bind its ligand (fluorescence-monitored ligand-binding assays) indicating

Table 1 Summary of the expression, solubilization and refolding conditions for three efficiently refolded GPCRs, the OR5 receptor,⁴³ the BLT1 receptor²⁷ and the 5-HT4a receptor.³⁴ CHS: cholesteryl hemisuccinate, CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, DDM: dodecyl maltoside, DMPC: dimyristoyl-phosphatidylcholine, GST: glutathione S-transferase, KSI: ketosteroid isomerase, IB: *E. coli* inclusion bodies, LDAO: lauryl-N,N-dimethylamine-N-oxide, POPC: 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine and POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]. The lipid and/or lipid/detergent weight ratios used for refolding are indicated

Receptor	Expression	Solubilization	Refolding method	Detergent and/or lipid	References
OR5	as IB GST fusion	sarcosyl	matrix-assisted (Ni-NTA)	POPC/POPG (4:1)	43
BLT1	as IB T7-tag fusion	urea/SDS	matrix-assisted (Ni-NTA)	LDAO	27
5-HT4a	as IB KSI fusion	urea/SDS	matrix-assisted (Ni-NTA)	CHAPS/DMPC/CHS (2:1:0.02)	34

that, under these conditions, the receptor was properly folded. Subsequently, mixed micelles, composed of dodecyl maltoside (DDM) as the detergent, and of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) as the lipids, were added and the detergent was removed by treatment with hydrophobic beads. Under these conditions, the protein was stabilized into a fully active state as assessed by photoaffinity labeling. In the case of the BLT1 receptor, the best refolding yields were achieved with lauryl-*N,N*-dimethylamine-*N*-oxide (LDAO) as a detergent. For BLT1, different detergents were tested and the best yields were systematically achieved from those with a long alkyl chain, *i.e.* above C12. The observation of a prominent role of the alkyl chain length on BLT1 refolding is reminiscent of what had been previously observed with rhodopsin, where detergents with long alkyl chains (above C10) stabilized the protein better than detergents with shorter chains.⁴⁶ It is to be noted that BLT1 and OR5 (see above), were first refolded in solutions containing only detergents. However, in this case, subsequent reconstitution of the receptor in a detergent/lipid mixed micelle or in a lipid vesicle dramatically increases the stability of the isolated BLT1 receptor in solution (Banères, unpublished data). This suggests that, even if the detergent can mimic the membrane environment for receptor refolding, the presence of lipids and maybe specific lipid/protein contacts, are essential to ensure a full stabilization of the protein in solution. Finally, the 5-HT4a receptor was stabilized in a functional conformation only in the presence of mixed detergent/lipid micelles. The micelles in this case were composed of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dimyristoyl-phosphatidylcholine (DMPC) and cholesterol (CH) (see Table 1). In contrast to what had been observed with BLT1, the 5-HT4a receptor could not be refolded in the presence of a detergent only, emphasizing the importance of lipids for the stabilization of the receptor conformation. Also, the presence of CH was required not only for increasing the refolding yields of the 5-HT4a receptor, but also to increase the long-term stability and optimize the ligand-binding properties of the receptor in solution (see Figure 2). The differences in the detergent requirements for stabilizing the functional conformations of the OR5, BLT1 and 5-HT4a receptors indicate that each protein may be a specific case in terms of detergent and lipid requirements for refolding. In the absence of more published data, it is difficult to infer a general rule as to which are the most appropriate detergent and lipids for GPCR refolding. The most straightforward strategy to find suitable folding conditions seems therefore to test different detergents and detergent/lipid mixtures in a systematic way.

Besides the step involving the search for a detergent that could promote receptor folding, the data obtained for the receptors cited above emphasize the fact that one of the main problems, when screening for the best conditions for protein folding, is to determine the yield of functional protein. This is easier to achieve for membrane proteins that display well-defined ligand-binding properties. In the case of OR5, BLT1 and 5-HT4a, the main criterion used to assess the correct refolding was the ligand-binding properties of the reconstituted receptor. It is to be noted in this context that one must be cautious while using an agonist to determine if the receptor has been successfully refolded. In the case of an isolated receptor, the agonist affinity may be significantly lower in the absence of G-proteins. Receptors isolated from

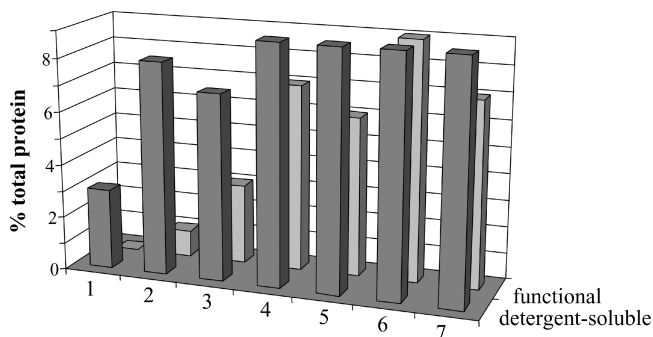


Figure 2 Percentage of detergent-soluble and functional (i.e. competent to bind antagonist) 5-HT_{4a} receptor after refolding in different detergent and/or detergent/lipid mixtures. (1) octyl glucoside, (2) LDAO, (3) CHAPS, (4) CHAPS/DMPC (detergent/lipid ratio 2:1), (5) CHAPS/DOPC (2:1), (6) CHAPS/DMPC/CHS (2:1:0.02), and (7) CHAPS/DOPC/CHS (2:1:0.02) (see legend to Table 1 for the abbreviations of the detergents and lipids)

membrane fractions may still be bound to endogenous G-proteins, and hence display higher agonist affinities. Other parameters can also be used to assess correct folding of isolated GPCRs. These include the ability of the refolded receptor to interact with and/or to activate intracellular partners, such as G-proteins or arrestins.^{47,48} Determining the amount of correctly refolded protein is more difficult when no biological assay is available. Centrifugation or filtration can remove precipitates resulting from protein aggregation, but solubility cannot be used as a stringent criterion simply because partially folded non-active intermediates can be soluble in the presence of detergents. Another method that has been largely applied to the refolding of globular proteins is limited proteolysis. Partially folded intermediates are assumed to be more susceptible to limited proteolysis than the fully folded protein. However, despite this method having successfully been applied to globular proteins,³¹ one must be cautious when working with membrane proteins, since inaccessibility to the protease could simply be due to the masking of the cleavage sites by the detergent and not to the correct folding of the protein. This method has nevertheless been successfully applied to membrane proteins, but rather to test, for example, for correct insertion in a lipid membrane.^{49,50} Spectroscopic methods, such as CD or fluorescence, that give access to the structural characteristics of the protein, can also be used to monitor refolding. CD will provide the secondary structure content of the protein,⁵¹ whereas tryptophan fluorescence spectroscopy can be used to detect folded protein, since unfolded conformations, folding intermediates and fully folded proteins may be distinguishable in their respective spectra.⁵² However, one must again be cautious while using these criteria to assess for correct membrane protein folding. For example, a good CD profile does not mean that the protein is well folded. If one considers the two-stage model for protein refolding, a possibility is to get a partially folded protein where the secondary structure elements are formed so that its far-UV CD properties are close to those of the fully folded protein, but where the intramolecular helical contacts are not properly established so that the receptor

is not able to bind its ligands. One must also keep in mind that even in harsh detergents, such as SDS, helical membrane proteins, bacteriorhodopsin or GPCRs usually can retain a certain amount of secondary structure. A good alternative in this case could be the use of CD in the near-UV regions, rather than in the far-UV regions. Indeed, the near-UV region is sensitive to the three-dimensional folding of the protein and is therefore likely to be affected by the packing of the transmembrane helices.^{27,34} As for fluorescence, it can be difficult to assess correct refolding in the absence of a reference spectrum of the functional protein. In a general manner, for most of the methods described above, one of the main problems indeed arises from the absence of well-folded protein to be taken as a reference due to the low abundance of most GPCRs in cellular membranes. Finally, as a consequence of the possible occurrence of detergent-soluble misfolded proteins, a proper way to purify the functional receptor is also crucial. In this context, the best method seems to be, whenever possible, affinity chromatography with an immobilized ligand column, since the main goal of this purification step is to discriminate between active and inactive receptors.

To give an example emphasizing the importance of a functional assay for assessing receptor refolding, we can consider the case of the 5-HT_{4a} receptor. When refolding was carried out with only detergents, two different protein fractions were recovered after the refolding step. The first one, highly aggregated, was simply removed by centrifugation on a sucrose gradient. The second one was totally soluble in the detergent-containing solution and its secondary structure properly recovered as assessed by far-UV CD. However, only a small amount of receptor in this fraction was able to bind a 5-HT_{4a} antagonist ligand, as assessed by direct ligand-binding experiments (Figure 2). It is only after adding lipids in the refolding buffers that a fully functional protein fraction was recovered.

5 Conclusion

Membrane protein refolding, in particular GPCR refolding, has been the focus of intensive work during the past years. This is due to the possible implications of receptor misfolding in some diseases as well as to the interest of GPCR refolding in the context of high-yield protein production for structural studies. Indeed, the increasing number of reports of production of GPCRs in *E. coli* inclusion bodies makes refolding a central step for producing functional receptors. Although further development of the techniques and refolding systems are still required, an understanding of the process is being gained. The possibility to study the refolding of model proteins, such as bacteriorhodopsin, and now a few GPCRs, will certainly provide us with a more detailed model of the refolding mechanism. It is likely that future success in refolding more membrane proteins and/or reaching higher refolding yields *in vitro* will depend on how we understand the structural properties of the membrane proteins, as well as the way they interact with their lipid environment. In particular, some work will certainly have to focus on the factors that influence the interactions between helical domains in the membrane protein since this is, at least in our hands, the limiting step for reaching efficient refolding of all the GPCRs we have studied so far. Such fundamental work on receptor refolding will without doubt

help in finding the factors that currently limit high-level *in vitro* refolding of GPCRs, especially since high-level expression of “unfolded proteins” seems now somehow to be ensured, at least in the bacterial system.

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