New Tools for Membrane Protein Research

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Abstract: The last five years have seen a dramatic increase in the number of membrane protein structures. The vast majority of these 191 unique structures are of membrane proteins from prokaryotic sources. Whilst these have provided unprecedented insight into the mechanism of action of these important molecules our understanding of many clinically important eukaryotic membrane proteins remains limited by a lack of high resolution structural data. It is clear that novel approaches are required to facilitate the structural characterization of eukaryotic membrane proteins. Here we review some of the techniques developed recently which are having a major impact on the way in which structural studies of eukaryotic membrane proteins are being approached. Several different high throughput approaches have been designed to identify membrane proteins most suitable for structural studies. One approach is to screen large numbers of related or non-related membrane proteins using GFP fusion proteins. An alternative involves generating large numbers of mutants of a single protein with a view to obtaining a fully functional but highly stable membrane protein. These, and other novel techniques that aim to facilitate the production of protein likely to yield well-diffracting crystals are described.

Keywords: Eukaryotic membrane proteins, high resolution structure determination, high throughput pipelines, conformational thermostabilization, aggregation, amphiphiles.

INTRODUCTION

The lipid bilayer of biological cells and organelles constitutes the dynamic barrier between the interior and exterior environments. Integral membrane proteins housed within the lipid bilayer are involved in a wide range of biological functions including respiration, photosynthesis, uptake of nutrients, efflux of waste products and toxins as well as mediating cellular responses to a wide range of biologically active molecules. In addition, it is estimated that 20-30% of eukaryotic open reading frames (ORFs) encode for α-helical membrane proteins [1,2]. The importance of these proteins to cellular and whole organism physiology has made them the subject of intensive study. Until relatively recently, understanding of almost all groups of membrane proteins was severely hampered by the lack of high-resolution structural information. However the last 10 years have seen a mini explosion in the numbers of membrane protein structures [3]. This has provided unprecedented insight into the mechanism of action of several groups of membrane proteins. However of the 191 unique membrane protein structures available (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html) only 47 are of eukaryotic origin with the majority of these obtained using protein from naturally abundant native sources and the remaining obtained using protein from recombinantly expressed sources. These numbers reflect the difficulty of obtaining high quality eukaryotic integral membrane protein (eMP) samples suitable for structural studies.

Whether the relatively high number of prokaryotic membrane protein structures can be used to fill the gaps in knowledge about eukaryotic membrane proteins was addressed recently by Granseth and colleagues [4]. Somewhat unsurprisingly, the conclusion of their analysis was that to an extent, prokaryotic membrane protein structures where available can be used to model eukaryotic homologues. In the absence of a prokaryotic homologue or where more definitive data is required, i.e., in almost all cases, then a high resolution structure of the eukaryotic protein is required.

The pressing need for these structures means that novel methods are required to address the problems associated with structural studies of eukaryotic integral membrane proteins (eMPs). This review outlines some of these problems and summarises some of the key methodological advances made in the last few years, which have been successfully applied to membrane protein structure determination.

EXPRESSION

Despite major effort, the expression of eMPs still represents a major bottleneck in the production of proteins for structural studies [5,6]. There are examples of successful expression of eMPs in *E. coli* [7,8] however to date there is only one high resolution structure of an eMP expressed in *E. coli* (human 5-lipoxygenase-activating protein [9]). More successful alternatives are the eukaryotic expression systems, particularly the yeasts, *Pichia pastoris* and *Saccharomyces cerevisae,* and insect cell based systems. These have a number of advantages over *E. coli* in that they are capable of post-translational modifications and have lipid contents closer to higher eukaryotes [10]. A number of membrane proteins expressed in *P. pastoris* have yielded high resolution structures including the mammalian voltage-dependent potassium channel [11], the plant aquaporin [12] and the human LTC4 synthase [13] and most recently the mouse

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1389-2037/10 $55.00+.00

ABC multidrug transporter, P-glycoprotein [14]. *S. cerevisae* has been used to produce protein which ultimately yielded the high resolution structure of P-type proton pump from *Arabidopsis thaliana* [15] and diffracting crystals of rabbit Ca^{2+}ATPase SERCA1a [16]. Insect cell based expression systems have had spectacular successes for the production of G-protein coupled receptors (GPCRs) for structural studies, so far yielding structures of 3 independent proteins, the human b_2-adrenergic receptor [17,18], the turkey b_1 adrenergic receptor [19] and the human adenosine A_{2A} receptor [20]. In addition, insect cells were also used to produce the chicken voltage dependent acid sensing ion channel which was solved to 1.9 Å resolution [21].

The majority of researchers working in the area of eukaryotic membrane protein structural determination use one of these three systems for protein production. Other systems are available including the Semliki Forest Virus mammalian cell expression system [22], which has had particular success in high level expression of GPCRs [23] and an alternative bacterial system using the Gram positive bacterium *Lactococcus lactis* [24]. Neither system has yet resulted in a high resolution structure but it is unclear whether this is due to inherent unsuitability of the systems for large scale production of eMPs for structural studies or the fact that these systems are not as extensively utilized as the yeast and insect cell systems. It will be interesting to see if in the future, these systems prove viable alternatives to yeast and insect cell cultures. An alternative approach is to attempt expression in as many different systems as possible as described for the human serotonin receptor [25] .

Although some researchers are performing careful, rational optimization of the existing systems with a view to maximizing quality and quantity of the target proteins [26], not many major advances have been made in terms of expression of eMPs in the last 5-10 years. One exception to this is the development of cell-free expression systems for the production of membrane proteins [27,28]. This type of approach involves in-vitro production of proteins outside intact cells from a DNA or mRNA template using a basic set of biological building blocks prepared from cell lysate [29]. A wide range of cell lysates are available and it has been shown that those from eukaryotic sources, e.g. rabbit reticulocytes, are capable of post-translational modifications [30], suggesting these may be suitable for production of eukaryotic membrane proteins. By its very nature, the system removes the problem of cytotoxicity and also simplifies protein isolation as the number of contaminant proteins is markedly reduced. A further advantage of this system is the high level of control a user has, in terms of modifying media components to both increase the stability of the expressed proteins (e.g., addition of protease inhibitors, lipids, co-factors) and to effectively, efficiently label for analysis by NMR [31] or X-ray crystallography. A major advantage for membrane proteins is that the expressed MPs are maintained in a soluble state in detergent micelles post-translationally since there are no native membrane environments for insertion [32]. There are many examples of the production of functionally active prokaryotic and eukaryotic MPs in cell free systems [27]. In addition, and most promisingly, it has proved possible to prepare selenomethionine labeled sample of a bacterial multidrug resistance transporter, EmrE. This protein was successfully crystallized and these crystals contributed to structure determination [33]. As ever, work on eMPs lags behind but efforts are currently underway to exploit this system effectively for structural studies.

PIPELINE APPROACHES FOR THE RAPID IDENTIFICATION OF EMPS SUITABLE FOR STRUCTURAL STUDIES

Homologue screening as a means to identify membrane proteins suitable for structural studies has been utilized effectively a number of times, for example in the case of the bacterial Sec translocon [34]. In this case, researchers started with 10 bacterial homologues and focused their efforts on the protein which expressed to the highest level and was most stable in a range of detergents [34].

Larger scale operations for membrane proteins based on the soluble protein structural genomics efforts have been much less successful as it is much more difficult to streamline operations using single expression constructs, expression systems and purification strategies as has been described for soluble protein work Parallelisation approaches attempting high-throughput screening in a number of different expression systems and using a wide range of expression constructs have been attempted and produced large quantities of data but the overall process has proved somewhat cumbersome and extremely expensive. The development of so-called pipelines approaches, analogous to the drug discovery pipelines utilized extensively by the pharmaceutical industry may have gone some way to rationalizing the identification of eMPs for structural studies. Like the drug discovery pipelines these approaches have a series of built in quality control checkpoints. Any protein which fails to reach a set of criteria is excluded from the target list. As in the case of drug discovery, the overall aim is to remove less suitable targets as early on in the process as possible, diverting more time and resources to the most promising ones. Adequate numbers of homologues must be identified and robust checkpoints established.

One heavily used approach is the *S. cerevisae* GFP pipeline [35,36]. The expression vector incorporates a galactose inducible promoter and a tobacco etch virus (TEV) cleavage site followed by the gene coding for a C-terminally His tagged green fluorescent protein (GFP). Homologous recombination is utilized to rapidly generate expression constructs where the gene of interest is inserted upstream of the TEV cleavage site. This results in a construct whereby the recombinantly expressed target protein is fused to a C-terminally located GFP (Fig. **1A**). The His tag on the GFP facilitates purification while the TEV cleavage site allows efficient removal of the GFP resulting in an almost native protein. Protein expression is performed in a protease deficient *S. cerevisae* cell line [37] using small scale cultures (10 ml). The key to this system is the presence of the GFP, which allows rapid assessment of the expression levels by simple fluorescence measurements. These measurements represent the first checkpoint on the pipeline-any protein expressing at less than 1 mg/L can be subjected to simple optimization. If the required expression level is not reached however, the target is removed from the pipeline. Integrity of the expressed protein can be assessed using in-gel fluores-

cence, a convenient alternative to Western blot analysis (Fig. **1B**) which is much quicker and does not require the use of expensive antibodies. A protein which shows multiple bands on a gel may be subject to either degradation and/or aggregation and may warrant either further investigation or removal from the pipeline. Further preliminary analysis, requiring larger scale culture (500 mL), can be performed using fluorescent size exclusion chromatography (FSEC [38]), a technique which allows assessment of the aggregation status of solubilised GFP tagged proteins (Fig. **1C**). Size exclusion chromatography (SEC) is carried out as normal but the samples can be either collected in an adapted fraction collector suitable for analysis by a 96-well plate fluorimeter or an in-line fluorescence detector. Since only the fluorescent proteins are detected it is possible to analyse crude solubilised samples in this way (Fig. **1C**). Several different detergents can be assessed to give an indication of the stability of the target protein in both long chain detergents e.g., *n*-dodecyl-β-D-maltopyranoside (DDM) and shorter chain detergents potentially more suitable for crystallization trials e.g., *n*-nonyl-β-D-maltopyranoside (NM). Proteins which aggregate heavily (detected by an abnormally large void peak) or degrade (detected by an abnormally large GFP peak) can be excluded from the pipeline or subjected to further screening. A standard purification protocol is used for isolation of the proteins which has been tried and tested for a range of eMPs [36] prior to crystallization trials. The proteins are submitted to Ni^{2+} immobilized metal affinity chromatography (IMAC) followed by TEV protease cleavage to remove the GFP. The target protein is then separated from both the His-tagged GFP and the His tagged TEV protease by reverse IMAC. A final polishing step of SEC is also performed, which gives an indication of the aggregation status of the protein. The approach has so far yielded >30 pure eMPs in a form suitable for structural studies [36].

One clear potential criticism of such a method is that the protein assessed in the early stages is a GFP fusion protein and therefore not truly representative of the final protein sample. However the careful analysis carried out by Newstead and colleagues [35], demonstrates that the preliminary analysis is a reasonable indicator of the suitability of the target protein for further studies. A DDM-solubilised crude protein sample that exhibited aggregation during FSEC, also showed aggregation after purification and removal of the GFP tag. The same trend was observed for all the successfully purified examples where the absence of aggregation during FSEC analysis correlated well with the production of pure, monodispersed protein. However even using this system, there are instances where proteins aggregate heavily upon concentration to 10 mg/ml. It is very difficult to predict how any protein behaves at high concentrations without first isolating large quantities of that protein. In this case alternative strategies can be utilized in order to screen for buffer conditions which maintain the protein in a monodispersed state (See screening for aggregation below).

An alternative to the GFP pipeline has been developed by Robert Stroud's group [39]. In this case 384 *S. cerevisae* membrane proteins predicted to have three or more transmembrane domains were used as targets. As with the GFP system, ligation independent cloning techniques are used to clone the gene of interest into a vector incorporating the galactose inducible GAL1 promoter, an N-terminal FLAG tag and a PreScission 3C protease cleavage site, upstream of the gene of interest and a C-terminal thrombin protease cleavage site followed by a His tag. The gene of interest is cloned between the protease cleavage sites. This method proceeds directly to medium scale culture (500 ml) and measurement of both expression level and solubilisation efficiency in parallel. Both are assessed by semi-quantitative Western blot analysis using antibodies against both the FLAG and His tags. The checkpoints in this case are the presence of a Western blot signal indicating an approximate expression level and at least 50% recovery of DDM solubilised protein following high-speed centrifugation. Targets not meeting these criteria are removed from the pipeline. A straightforward purification protocol utilizing IMAC followed by SEC is used to both isolate and further characterize the proteins. Proteins yielding less than 0.5 mg/L after IMAC and those exhibiting high levels of aggregation during SEC are also removed from the pipeline. This approach rapidly identified a sub-population of targets (~25%) suitable for further, more intensive study. The approach was developed using eMPs from S. *cerevisae*, however application of the method to human MPs confirms the validity of the method to production of MPs from higher eukaryotic sources.

The possibility for fully quantitative analysis is one of the major advantages of the GFP pipeline however the Stroud approach is faster, simpler and requires very little sophisticated equipment. It is possible to easily obtain more information about the target proteins using the GFP pipeline, for example localization of the expressed protein; however it is not clear at this point whether that information is required to identify those targets suitable for further studies. In addition, the Stroud approach is not constrained by the requirement that GFP must be localized in the cytoplasm thus limiting its use to proteins with an intracellular C-terminus. Although it has been demonstrated that the large majority of membrane proteins are likely to have an intracellular C-terminus [40]. Overall both approaches are valid ways of rapidly identifying targets in the earlier stages of the process where the functional significance of a given protein only becomes a major consideration when that protein is shown to be suitable for structural studies. Both have high success rates in terms of producing crystallization quality protein (at least 90% pure and in mg quantities). However the identification and purification of these targets is not the end of the screening process since painstaking optimization of sample buffer conditions and crystallization conditions is still required for each individual target. The two approaches are based on the same principle that some eMPs are inherently more suitable for structural studies than others, and it is just necessary to sift through many unsuitable targets before finding the true gems.

ANTIBODY APPROACHES FOR GPCRS

In contrast to the high-throughput pipelines designed to screen a wide range of related targets, approaches focused much more on individual target proteins have been successfully applied to the structure determination of GPCRs. The GPCRs are key mediators of cellular responses to a wide range of biologically active molecules including hormones, neurotransmitters and approximately 50% of all available

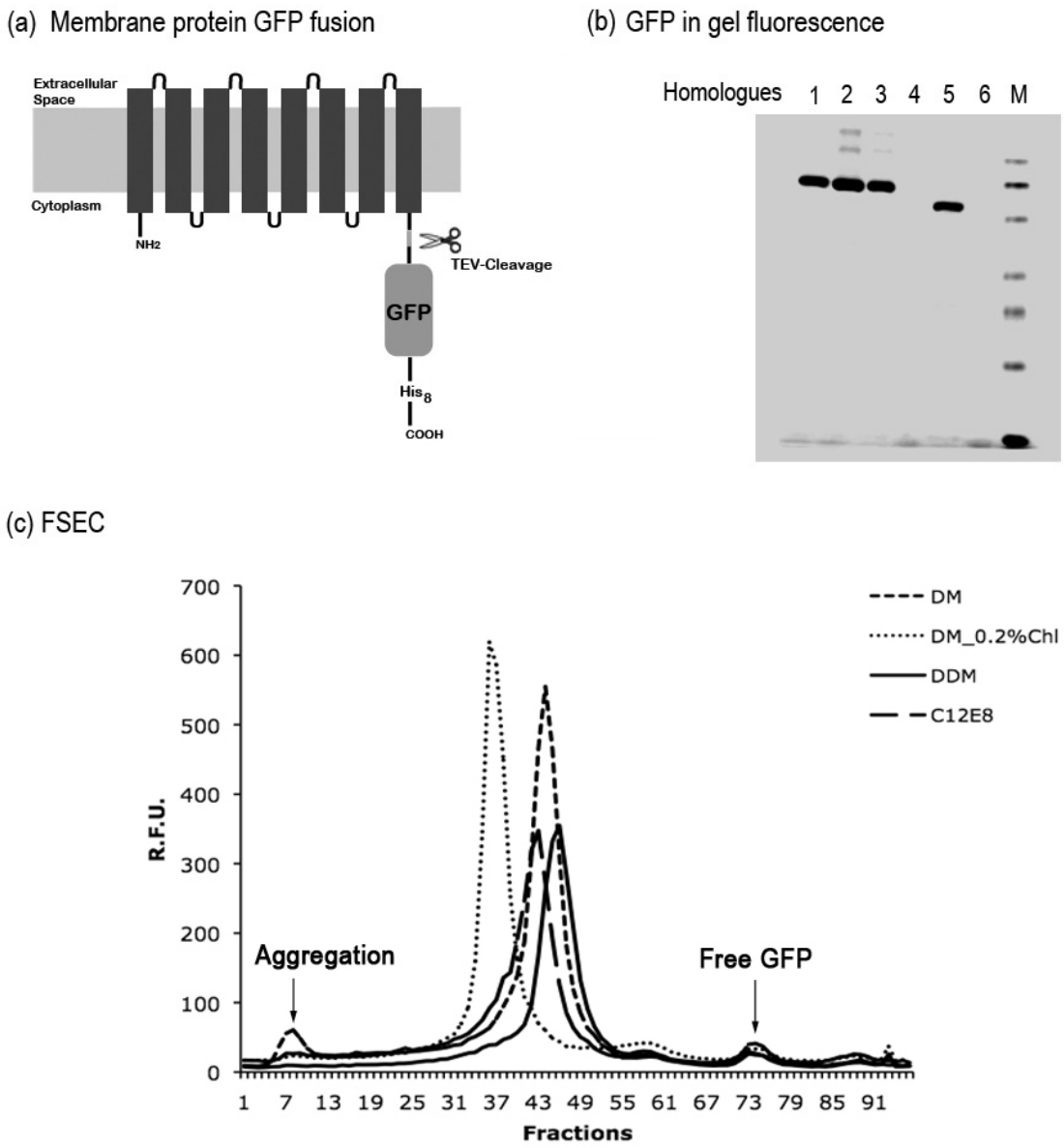

Fig. (1). (a) A topographical representation of a MP-GFP fusion protein. Purification of the fusion protein using IMAC is followed by cleavage with TEV protease to remove the GFP. The untagged target protein is separated from His-tagged GFP and His-tagged TEV protease by a further IMAC step. **(b)** In-gel fluorescence analysis of eukaryotic membrane proteins expressed as fusions with GFP. Crude membrane preparations containing the GFP-fusion proteins were separated on 10% Tris-Glycine SDS-PAGE. Visualization by in-gel fluorescence confirms the presence of full-length MP-GFP fusion proteins for 4 of the 6 tested proteins. **(c)** FSEC profile of a eukaryotic MP-GFP fusion protein solubilised in DDM, DM, DM +0.2% Cholesterol, and $C_{12}E_8$. All detergents yield a monodispersed solubilisation profile although it is possible that there is higher oligomer formation in the presence of cholesterol. The expected positions of peaks corresponding to aggregated protein and free GFP are indicated on the FSEC profile.

drugs [41]. For many years structural studies on these molecules had lagged behind those on other classes of membrane proteins including transporters and ion channels. The first major success came in 2000 with the high resolution structure of bovine rhodopsin [42], a non-typical GPCR with an intrinsic ligand which changes conformation upon interaction with a photon of light. This induces a further conformational change in the GPCR which activates the G-protein and initiates an intracellular signaling cascade. This structure provided a template for other studies and indeed drug design [43]. However the very features of rhodopsin that made it amenable to structural studies made it of limited use for understanding the precise details of ligand-receptor interactions for all other GPCRs. High resolution structures are needed for a wide range of such receptors.

The major problem of low yields of functional recombinant expression of GPCRs had been overcome in a number of cases using a variety of expression constructs and systems [7,8,44,45]. In addition, there were examples of GPCRs being purified to high levels of homogeneity and exhibiting long term functional stability [45-47]. The research also

benefitted from the ready availability of high affinity ligands to produce a more conformationally homogeneous sample. However despite the production of protein apparently suitable for structural studies no well-diffracting crystals were obtained. Almost simultaneously, three different approaches produced breakthroughs resulting in high-resolution protein structures of two different but related receptors.

The first structure, a partial structure of the β_2-adrenergic receptor [18], was solved in complex with an antibody fragment (Fig. **2A**). The antibody bound to the third intracellular loop, stabilising the loop and providing the principal surface for crystal contacts within the lattice. The structure only revealed details of about two thirds of the protein, mainly the intracellular loops and the cytoplasmic side of the transmembrane (TM) helices. The extracellular ends of the TM helices and the extracellular loops were too disordered to allow the structure to be resolved in these regions. Nevertheless, this first structure gave insights into the overall architecture of the β_2-adrenergic receptor and allowed comparison with the structure of rhodopsin [18]. The antibody approach, to extend the hydrophilic domain of membrane proteins, has been used successfully for a number of both prokaryotic and eukaryotic membrane proteins [48-51]. Despite these successes, the traditionally long and expensive preparation and screening process for the antibodies with no guarantee of success has made this approach unattractive to many working in the field. Novel approaches using designed ankyrin repeat proteins (DARPins) as alternative co-crystallisation agents have been successfully applied to the high resolution structure determination of the bacterial multidrug resistance protein, AcrB [52]. Ankyrin repeat motifs are present in a wide range of naturally occurring proteins with diverse cellular roles, and are best characterized in terms of their importance in protein-protein interactions. The sequence motif has a defined architecture with a relatively small number of residues responsible for protein-protein interaction. Protein libraries have been generated using phage display and ribosome display technology which allow the rapid generation of a large number of potentially high affinity binding proteins. The proteins vary in the amino acid composition of the protein-protein interaction sites but have a maintained structural fold. These libraries can be screened for high affinity binding to a target protein with the best binders being used as co-crystallisation agents [53]. The relative ease of generation and screening of the DARPins makes them an attractive alternative to monoclonal antibody fragments. Currently there are no examples of eMPs crystallized in complex with a DARPin, however these binders have significant potential for the future.

THE T4-LYSOZYME FUSION APPROACH

The second approach to structural determination of a GPCR involved removal of disordered regions of the human β_2-adrenergic receptor (intracellular C-terminal domain and third intracellular loop) and insertion of a highly ordered protein, T4 lysozyme, into the third intracellular loop [54]. This chimeric protein expressed well in insect cells, was shown to be highly functional and ultimately yielded well diffracting crystals and a high resolution structure [17]. The presence of the T4 lysozyme did not significantly alter the conformation of the receptor as confirmed by a comparison with the structure of the partial protein in complex with the antibody fragment [18]. The major flaw in the approach is the removal of key domains important for G-protein coupling and downstream signaling events. However the structure obtained provided the first detailed insight into the ligand binding pocket of the b_2-adrenergic receptor. Subsequent studies using the same receptor construct have also provided insight into the role of cholesterol in the structural integrity of the receptor [55]. This approach has since been successfully applied to the structure determination of another GPCR, the human adenosine A_2 receptor [20] suggesting that this method may be suitable for a wide range of GPCRs.

ALANINE SCANNING MUTAGENESIS

The third and final approach involved mutagenesis of the target receptor, in this case the b_1 adrenergic receptor from turkey, with a view to generating a mutant receptor with increased thermostability in solution and thus more suitable for structural studies. A similar approach had previously been used for the diacylgycerol kinase (DGK), an integral membrane enzyme from *E.coli*. Random mutagenesis was used to generate DGK mutants, which were assessed for enzyme activity before and after heat treatment. The most thermostable substitutions were combined to produce a quadruple mutant which had a half-life of 35 min at 80°C when solubilised in *n*-octyl-β-D-glucopyranoside (OG) compared to a half-life of less than 1 min for the wild-type protein [56].

Researchers began the study with a turkey β_1 adrenergic receptor construct which had already been significantly modified to improve expression in insect cells, facilitate purification and eliminate proteolytic sensitivity [45]. The resultant construct (residues 34-424) was submitted to alanine scanning mutagenesis [57] whereby 318 amino acid residues of the truncated turkey β_1-adrenergic receptor were mutated to an alanine, except when an alanine was present in the native protein, in which case a leucine substitution was made. Each of the mutants was expressed in *E. coli* for screening purposes. The thermostability of each mutant protein was assessed by radioligand binding analysis before and after heating to 32°C (T_m of the wild-type receptor) and compared with the wild-type protein. Whilst most of the mutations had either no effect or a negative effect on thermostability some resulted in increased thermostability. Certain combinations of the thermostabilising mutants were shown to further increase thermostability and the best construct, m23, containing a combination of six point mutations, had an apparent T_m 21°C higher than the wild-type receptor. Interestingly, unlike the wild-type form which was only stable in DDM, the m23 mutant was stable in short chain detergents and in the absence of ligand was preferentially in the antagonist bound conformation leading to greater homogeneity of the protein sample. The overall improved stability of m23 had a major effect on the ability of the protein to crystallise and this construct yielded well-diffracting crystals in the C8 detergent octylthioglucoside and ultimately a high resolution structure ([19]; Fig. **2C**). It should be mentioned that for structural studies, the m23 mutant was expressed in insect cells and not in *E. coli*.

This conformational thermostabilization process has been performed on two further GPCRs; the human adenosine A_{2A}

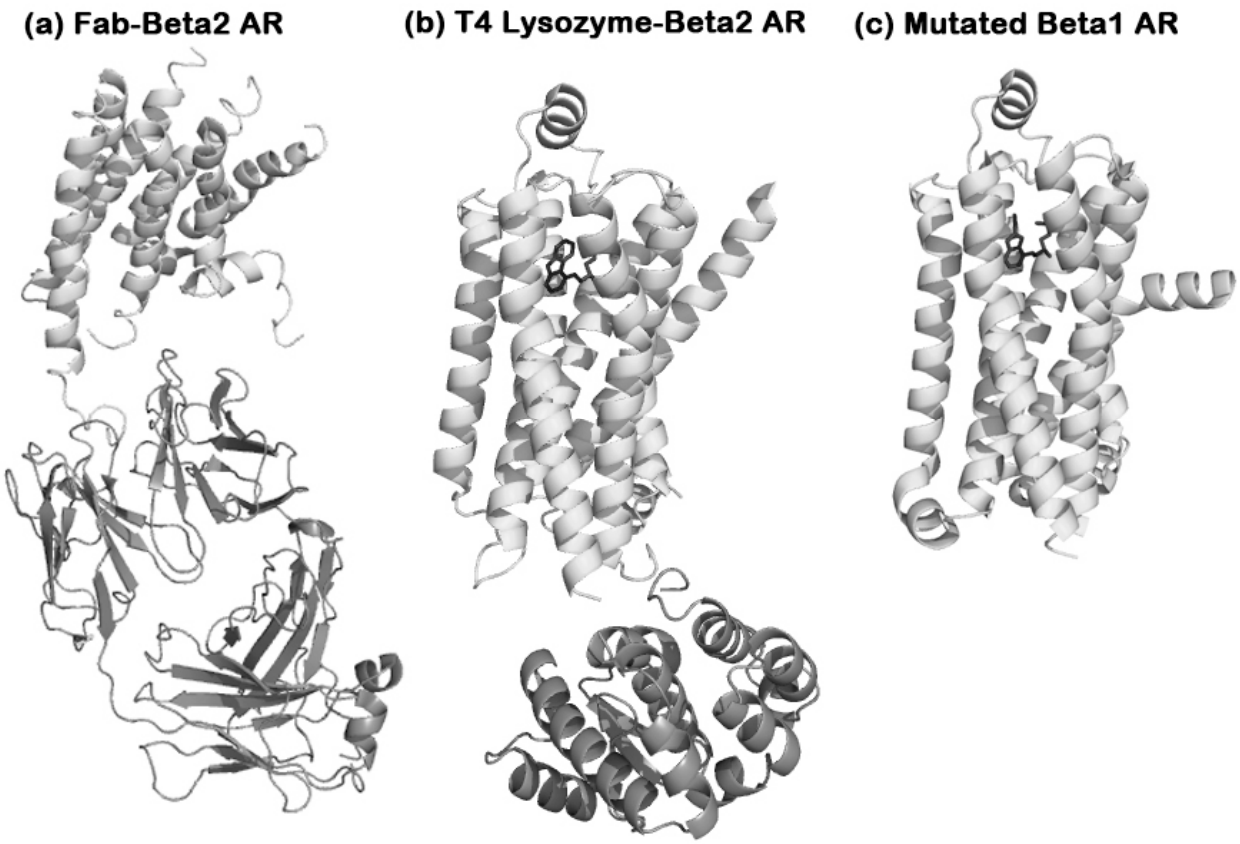

Fig. (2). Ribbon representations of three GPCR structures obtained using proteins stabilised in three different ways. **(a)** Human β_2-adrenergic receptor (light grey) fused with a T4-lysozyme (dark grey), ([17], 2RH1.pdb). The inverse agonist carazolol, bound in the ligand binding site of the receptor, is indicated as a stick model. **(b)** Structure of the human β_2-adrenergic receptor crystallized in complex with a Fab-antibody fragment (dark grey ribbons) fragment ([18], 2R4R.pdb). **(c)** Structure of the thermostabilised mutant β_1-adrenergic receptor from turkey ([19], 2VT4.pdb). The high affinity antagonist, cyanopindolol, bound in the ligand binding site of the receptor, is shown as a stick model.

receptor [58] and the rat neurotensin receptor [59]. As yet these studies have not resulted in high resolution structures but it will be interesting to see how widely applicable this approach is both to other GPCRs and other classes of eukaryotic membrane proteins. One interesting issue raised by this work is that it is not clear why mutating particular residues makes the receptors more stable. After completing the process with three separate receptors no pattern has emerged allowing prediction of sites where mutation would increase thermostability. Currently, at least, future work using this technique still requires extensive alanine-scanning mutagenesis together with a means for reliable assessment and comparison of thermostability and functionality of the mutants.

ASSESSMENT OF MEMBRANE PROTEIN STABILITY

As mentioned above, improved stability has proved critical to the successful crystallization of a number of eMPs. The recognition of this has led to the development of generic methods to assess the thermostability of a given membrane protein and to allow rapid and efficient screening of conditions which give stable, protein suitable for structural studies. The recent report of a fluorescent thermal stability assay specific for membrane proteins [60] has followed on from the successful application of such methods to soluble proteins. The basis of these assays is the use of dye molecules which bind to proteins as they unfold. Upon heating unfolding is detected as an increase in fluorescence as a result of the reaction between the dye and the unfolding protein. The dyes, e.g. Sypro orange, used for soluble proteins are not suitable for use with membrane proteins as they give very high background levels thought to be due to non-specific binding to detergent molecules and the hydrophobic regions of membrane protein molecules. The recently described approach [60] utilizes an alternative dye, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM)[60], a fluorochrome which reacts readily with the free thiols of cysteine residues. As described for the soluble protein work, as the membrane protein unfolds the normally buried cysteine residues become available to interact with the dye and the level of fluorescence gives an indication of the rate of unfolding. The CPM is virtually non-fluorescent in the unbound form and exhibits low non-specific binding to detergent and folded membrane protein. The assay procedure is very straightforward involving dilution of small amounts of protein (1-20 μg) in an appropriate test buffer, addition of the CPM dye and slowly heating the sample from low to high temperature in a fluorimeter, taking fluorescence measurements at regular intervals. The rate of unfolding in any

given assay condition can be easily compared with standard test conditions, e.g., NM compared to DDM.

The very specific interaction between the CPM and the thiol groups of cysteine residues is the major strength of the method however it is only possible to detect unfolding in those proteins containing cysteine residues. For proteins lacking cysteines alternative methods need to be used to assess thermostability or cysteine residues need to be mutated into the protein [60]. In addition the dye is not compatible with buffer conditions lower than pH 5.0 and higher than pH 8.0 again potentially requiring alternative methods. Despite these minor drawbacks the thermal stability assay is a very efficient method for comparing the stability of a given membrane protein in a range of different detergents, buffer and ionic strength conditions, additives etc. which requires very small amounts of protein and can be performed in a very short time [55]. These features make the assay an ideal method for high-throughput analysis of sample conditions more suitable for crystallization. Other techniques for analyzing the thermostability of integral membrane proteins include differential scanning calorimetry (DSC), circular dichroism (CD), UV/VIS spectroscopy and nuclear magnetic resonance (NMR). These methods can provide complementary assessment of thermostability however they usually require much larger amounts of proteins and are currently not well suited to high-throughput analysis.

SCREENING FOR AGGREGATION

Detergent-solubilised membrane proteins are very prone to non-specifically aggregate, particularly at the high concentrations required for crystallization trials. This is highly disadvantageous to downstream structural studies since non-specific aggregation of the protein reduces the likelihood of the formation of specific protein-protein interactions essential for crystal lattice formation. There are a number of methods to assess aggregation status but many of these are either low throughput or unsuitable for use with detergent solubilised samples. One recent development which allows both detection of aggregation and rapid screening of conditions to inhibit aggregation is the ultracentrifugation dispersity sedimentation (UDS) assay where ultracentrifugation of small (5 µL) volumes of purified, soluble membrane protein is combined with SDS-PAGE analysis to rapidly assess the degree of protein aggregation in high concentration (10-20 mg/ml) sample [61]. The novelty of the assay lies in the combination of these two standard laboratory techniques to assess the aggregation status of a protein in a number of different conditions simultaneously. The assay is based on the assumption that protein aggregates are orders of magnitude heavier than dispersed protein particles and can therefore be removed by sedimentation at high *g* forces. Protein samples taken before and after high-speed ultracentrifugation (~ 350, 000 *g*) are visualised on SDS-PAGE gels. Those samples which exhibit aggregation will show lower levels of protein in solution after ultracentrifugation [61]. The small volumes required mean that the UDS assay is economical in terms of both protein and detergent. It also allows assessment of the aggregation status of the protein sample at high concentrations, not requiring dilution of the sample or modification of the sample buffer as is necessary for SEC. This method has been used to both identify conditions which maintain a membrane protein in a monodispersed state at high concentration and to screen for detergents and buffer conditions suitable for optimisation of preliminary crystals.

DEVELOPMENT OF NEW DETERGENTS

The extraction of membrane proteins from the bilayer into detergent solubilized conditions is a prerequisite for structural studies. The best classical detergents for membrane protein research are usually nonionic and have the key feature of being able to extract membrane proteins with high efficiency whilst maintaining structural and functional integrity [62]. However protein-detergent micelles are particularly unstable, prone to aggregation and often exhibit a loss of protein function. The detergent molecules themselves are also disordered [63] and likely to inhibit the formation of highly ordered crystal lattice. In order to address these issues much effort has been directed towards the production of novel detergents that form more stable protein-detergent micelles, more suitable for protein isolation and crystallization [64-66]. The aim is to reduce the disorder introduced by the intrinsically flexible alkyl chains of commercial detergents and thus increase the chance of an ordered crystal lattice in all three dimensions. There are a range of such molecules, including amphipols [64] and tripod amphiphiles [66]. The amphipols (amphipathic polymers) were designed to form a tighter interaction with the membrane protein in order to reduce instability incurred through rapid association and dissociation of the detergent molecules. These have been shown to increase stability of some proteins. The major use of the amphipols may be as additives since their stabilizing effects seem to be greater when used in combination with detergents and lipids [65,67]. The so called "tripod amphiphiles'" (TPAs) are comprised of a tetrasubstituted carbon atom with one hydrophilic substituent and three hydrophobic substituents [68]. The tetrasubstituted carbon is thought to introduce extra rigidity through the reduction of conformational flexibility. This feature means that TPAs should be more stable than conventional detergents and this leads to the hypothesis that these molecules are more likely to contribute to an ordered crystal lattice [66]. Studies have demonstrated that TPA (Fig. **3**) gives greater solubilisation efficiency of bacteriorhodopsin (bR) from purple membranes than OG, Triton X-100 or DDM and more importantly TPA–solubilised bR was more stable [66]. Small crystals of TPA-solubilised bR have been obtained which diffract to 2.5 Å resolution [69]. This represents a small increase in resolution

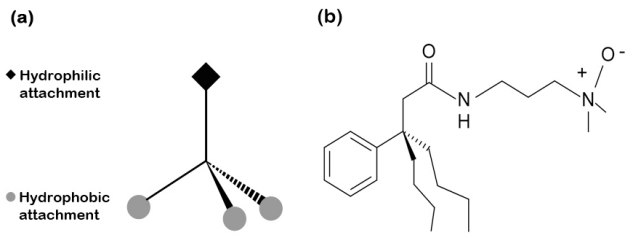

Fig. (3). Schematic representation of a tripod amphiphile. **(a)** The basic shape of a tripod amphiphile consisting of a tetra-substituted carbon atom with one hydrophilic substituent and three hydrophobic substituents. **(b)** The chemical structure of the tripod amphiphile that was successfully used to crystallise bacteriorhodopsin (bR).

compared to larger crystals of OG-solubilised bR. The increase in resolution is suggestive that TPAs may facilitate crystallization of membrane proteins either as alternatives to conventional detergents or as additives to crystallization trials. More research is required to further characterize the TPAs and to explore their true potential as alternatives to standard detergents.

CONCLUSION AND FUTURE PERSPECTIVES

Here we have summarized some of the key developments in the methodologies available to researchers working with membrane proteins. These advances have led to some spectacular success in the structure determination of eMPs, particularly the GPCRs. As described, the process of obtaining a high resolution structure is often as much an issue of identifying the unsuitable constructs, protein homologues and buffer conditions and excluding these from further studies as it is about identifying the optimal ones. Much effort has been put into developing techniques to make this process faster and more effective. While the number of eMP structures remains currently low it is anticipated that the long term effects of these advances will soon be felt as more eMPs suitable for structural studies are identified and studied in detail. Until then, it remains difficult to state which approaches will prove the most useful, although the production of T4 lysozyme fusion proteins holds great potential for the comparatively routine structure determination of GPCRs. One key issue remains the structure determination of multiple conformational states of membrane proteins. In the case of GPCRs, it is possible that such an issue may be addressed by for example, the use of specific ligands together with G-proteins as was described for the recent structure of squid opsin [70] or a combination of mutagenesis and inhibitors as has been used successfully used for prokaryotic transporters [71]. Alternatively other combinations, e.g., alanine scanning mutagenesis together with use of a T4 lysozyme fusion protein may be advantageous. In addition, the use of antibody fragments and DARPins are likely to be of use in improving the resolution of MP structures and thus the molecular details.

Whilst X-ray crystallography remains the technique of choice for structure determination of membrane proteins, other techniques are emerging. The very recent report of the low resolution structure of the human BK potassium channel using cryoelectron microsocopy is the first structure of a membrane protein in a lipid environment [72]. Clearly such a technique has great potential for providing insight into the specific roles of protein-lipid interactions and for elucidation of multiple conformational states.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the Biotechnology and Biological Research Council, the Defense, Science and Technology Laboratories and GlaxoSmithKline for support. This work was also supported by the European Commission under the FP7 EDICT project (Grant No. 201924). CA is supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Brazilian fellowship.

ABBREVIATIONS

eMP = Eukaryotic integral membrane protein

ORF = Open reading frame

DDM = *n*-Dodecyl-β-D-maltopyranoside

NM = *n*-Nonyl-β-D-maltopyranoside

OG = *n*-Octyl-β-D-glucopyranoside

IMAC = Immobilized metal affinity chromatography

TEV = Tobacco etch virus

SEC = Size exclusion chromatography

FSEC = Fluorescent size exclusion chromatography

GPCR = G-protein coupled receptor

UDS = Ultracentrifugation dispersity sedimentation

CPM = N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide

TPA = Tripod amphiphile.

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Received: June 09, 2009 Revised: September 22, 2009 Accepted: October 01, 2009

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