

Stabilizing membrane proteins through protein engineering

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Integral membrane proteins (IMPs) are crucial components of all cells but are difficult to study *in vitro* because they are generally unstable when removed from their native membranes using detergents. Despite the major biomedical relevance of IMPs, less than 1% of Protein Data Bank (PDB) entries are IMP structures, reflecting the technical gap between studies of soluble proteins compared to IMPs. Stability can be engineered into IMPs by inserting stabilizing mutations, thereby generating proteins that can be successfully applied to biochemical and structural studies when solubilized in detergent micelles. The identification of stabilizing mutations is not trivial, and this review will focus on the methods that have been used to identify stabilized membrane proteins, including alanine scanning and screening, directed evolution and computational design.

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Introduction

Integral membrane proteins (IMPs) are encoded by over 30% of all human genes [1] and are involved in critical cellular processes including cellular communication and sensing, molecular transport across lipid bilayers, biosynthesis and cell adhesion. Because many IMPs are located on the surface of cells, it is not surprising that they constitute the major class of drug targets, with 39 of the top 50 selling U.S. prescription drugs in 2010 acting on IMPs (<http://www.drugs.com/top200.html>). IMPs have specifically evolved to function in the hydrophobic environment of lipid bilayers, which they need to be removed from with detergents for biochemical and structural analysis (Figure 1).

Detergents are amphiphilic molecules containing a wide range of polar groups and, usually, a linear alkyl group,

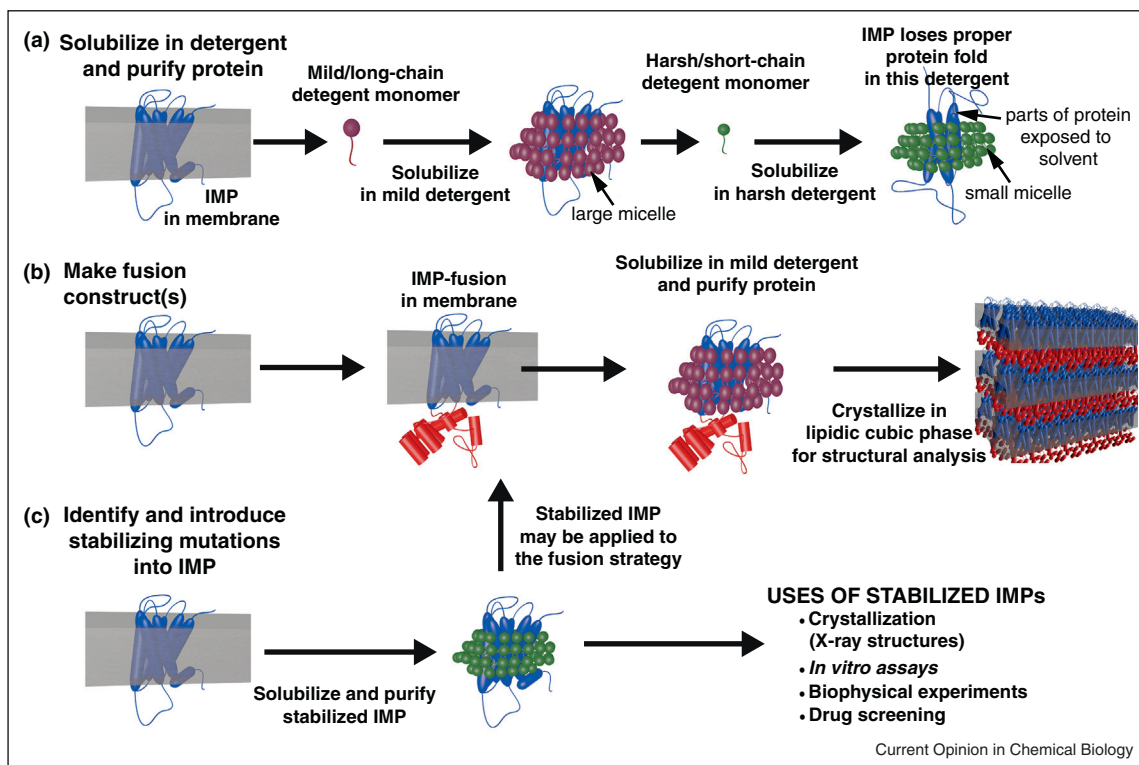
allowing them to form micelles in solution (Figure 1). IMPs solubilized in detergents become enveloped in detergent micelles, which partially mimic the membrane bilayer. A plethora of detergents exist for IMP purification: generally, milder detergents (long-chain detergents) are preferred, and they are uncharged and form large micelles that more closely mimic cell membranes (e.g., n-dodecyl-β-D-maltopyranoside (DDM)), while harsher detergents have shorter alkyl chains (e.g. n-octyl-β-D-glucopyranoside (OG)), and thus form smaller micelles that expose more of the IMP to the hydrophilic solvent (Figure 1). In IMPs without extensive extracellular domains, such as most G protein-coupled receptors (GPCRs), mild long-chain detergents tend to hinder the formation of protein–protein contacts and thus crystal formation, making the use of the harsh short-chain detergents a requirement for crystallization from detergents. Detergent molecules may also get access to internal cavities of the IMP, depending on the molecular structure of the detergent. All of these factors cause the environment within a detergent micelle to be different from the cell membrane, and thus IMP solubilization is typically denaturing, which has resulted in a large intellectual gap between our knowledge on the molecular aspects of IMP function in comparison to that of soluble proteins [2].

Much effort has been dedicated to methods that minimize the denaturation of IMPs during solubilization [3–5]. However, the inherent adaptability of IMPs can be harnessed to circumvent this problem in a different way. Because of their localization in a lipid environment, there are fewer protein fold classes for IMPs compared to soluble proteins [6]. The resultant protein families are, however, among the most evolutionary successful genes and have adapted to fulfill many biological functions [7–10]. Thus, rather than modify the conditions to suit the protein—which may not be sufficient to reach the goal—, several methods have now been established that allow the modification of the IMP to be more resistant to detergent solubilization. Here we will review and compare the protein engineering methods that have been applied to IMPs to find sequence modifications that confer increased IMP stability *without* disrupting biological functionality.

Stabilized IMPs through mutations

Pioneering work in the membrane protein stabilization field was carried out in the laboratory of James Bowie. Unlike many soluble proteins, the prototypical bacterial IMP diacylglycerol kinase (DGK), later found to be a domain-swapped trimer with three transmembrane helices per subunit [11], was found to be very tolerant

Figure 1



Rationale for stabilizing IMPs. **(a)** Many IMPs will denature when solubilized from the membranes of expressing cells with detergents, especially short-chain detergents, that form compact micelles, which are usually required for crystallization. **(b)** To avoid the need for short-chain detergents, IMPs can be fused to rigid, well behaving proteins such as T4-lysozyme (T4L). If stable enough, IMP-T4L fusion proteins can be solubilized and purified using mild, long-chain detergents and reconstituted into lipidic cubic phase (LCP) for X-ray crystallographic studies. **(c)** IMPs can be stabilized through mutation to generate variants that can tolerate detergent solubilization to varying degrees. Such stabilized variants can be used for vapor diffusion crystallization, T4L fusion and LCP crystallization, biophysical assays, *in vitro* assays and even drug screening assays.

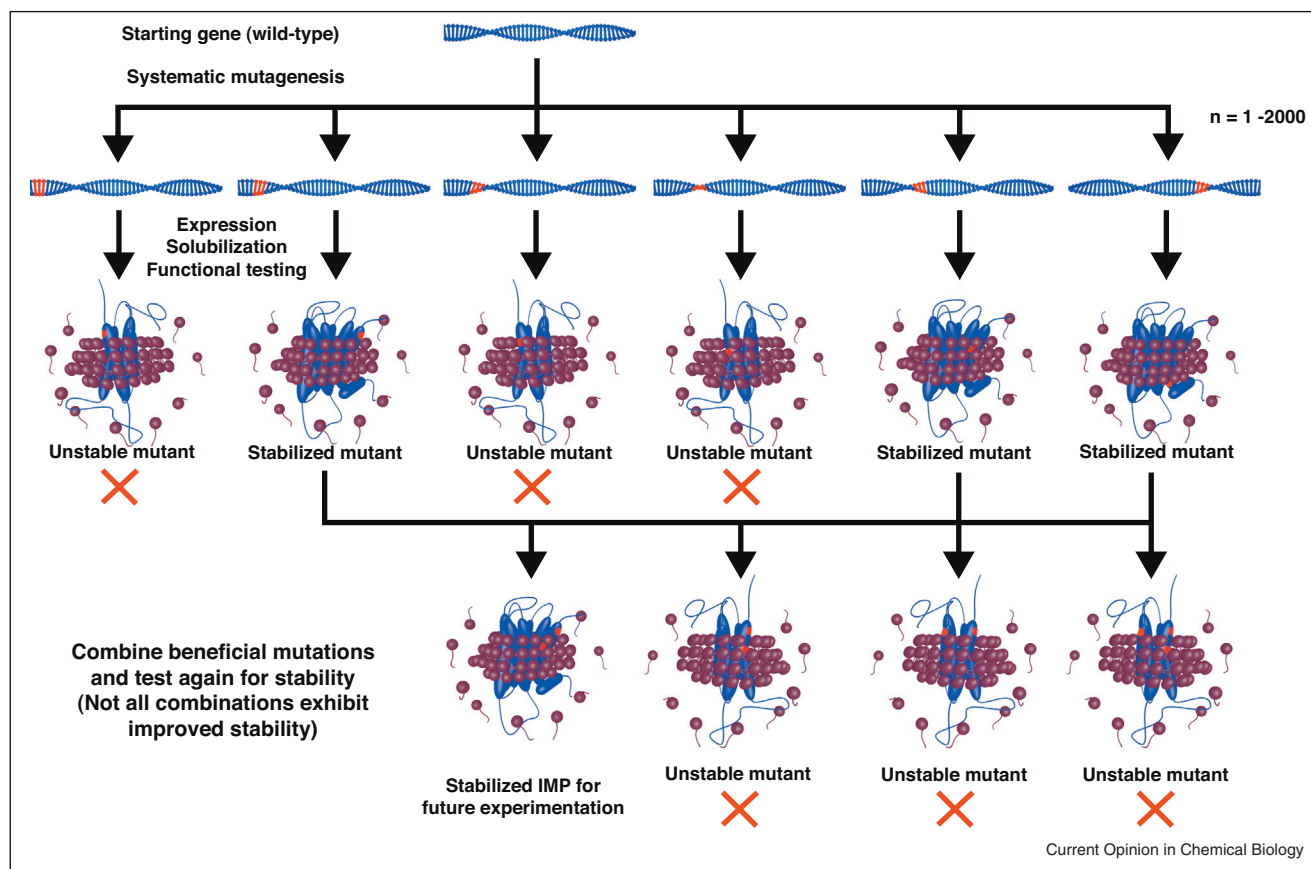
to mutations throughout its sequence [12]. A set of DGK mutants was then generated containing cysteine substitutions into 20 residues in the second transmembrane domain, two of which exhibited increased stability in detergent [13]. To our knowledge, these DGK variants, which exhibited half-lives of over 1 hour at 70 °C in harsh detergent, represent the first IMPs specifically engineered for improved stability in detergent. This study was built upon by making a randomly mutagenized library of DGK and screening over 1500 mutants for activity and a subset of these for stability in detergent [14]. Stabilizing mutations could then be combined with the two previously identified substitutions to introduce cysteine to generate even more stable versions of DGK. Key conclusions from this study were that the mutations found indeed increased the thermodynamic stability of DGK, but no obvious patterns could be discerned from the types of substitutions. Most importantly, stabilizing mutations were surprisingly frequent and could thus be easily identified from IMP mutant libraries. The general workflow for this method of stabilizing an IMP is depicted in Figure 2.

It was not long before one of the largest IMP research fields, that of GPCRs, began to delve into stabilization through protein engineering to aid in structural studies. A particular disulfide bond engineered into the visual GPCR rhodopsin (termed opsin in the apo-state) was found to greatly increase the stability of this IMP in detergent, measured by the ability of the receptors to bind 11-*cis*-retinal after solubilization, and aided in crystallization [15,16]. It should be noted that in the DGK mutants with substitutions to introduce cysteine leading to increased stability, these residues did not form disulfides, and the cysteine took the role of a hydrophobic amino acid, presumably improving the packing of the protein.

Alanine scanning mutagenesis

Bowie and coworkers were also the first to perform alanine scanning mutagenesis on a 7-transmembrane helix containing IMP, bacteriorhodopsin, to identify stabilizing mutations by monitoring the unfolding of the mutants upon exposure to SDS [17]. The main finding of this work was that a high frequency of alanine mutations,

Figure 2



Stabilizing IMPs through systematic mutagenesis. The IMP of interest is systematically mutated, typically by inserting a single amino acid substitution (alanine), generating as many single mutants as possible (<2000). These mutants are then individually expressed and tested for increased stability. Stabilizing mutations are then systematically combined, again in individual experiments, and the combination mutants are tested again for increased stability. Combinations that confer higher stability (usually not all of the combinations) can be used in downstream applications.

all within the second transmembrane helix of bacteriorhodopsin, resulted in receptors that were more stable when solubilized. This concept was then expanded upon and applied to several GPCRs by Tate and co-workers [18–21,22^{*}]. The typical workflow for these studies is to systematically generate mutants of the GPCR of interest, where each amino acid is mutated to alanine (leucine if the wild-type amino acid is alanine) and test each individually for increased stability in detergent, measured with radioligand binding assays. Positions that gain stability upon the introduction of alanine can be investigated further, by introducing other amino acids that may increase stability further [19]. Stabilizing mutations are then combined in various ways (again individually, clone by clone) and the most stable combination mutant is then used for downstream applications. GPCRs stabilized in this way have been used for fragment screening [23], biophysical mapping [24] and the crystal structures of two GPCRs have been published, to date the only ligand-activated GPCR structures to be solved with the receptor

solubilized in short-chain detergents [25–29]. A third receptor stabilized in this way, the rat neurotensin receptor (NTS₁) [20] was initially unable to be crystallized in detergents, but was recently solved using the T4 lysozyme (T4L) fusion and lipidic cubic phase crystallographic approach [30], which has typically been used to crystallize non-stabilized GPCRs [31–42]. It is important to note that it is not entirely clear if T4L fusion to GPCRs necessarily improves the stability of the IMP in detergent, however, the fusion may act as a conformational restraint and certainly aids in establishing crystal contacts [31,40]. Another mutant of NTS₁, obtained by directed evolution (see below), could be crystallized without T4L fusion in short-chain detergent (P. Egloff *et al.*, unpublished data).

The alanine scanning technique is to date the most established method for generating stabilized GPCRs for biochemical and structural studies (Table 1). Its success is likely due to the fact that IMPs have not been optimized

Table 1

Methods for stabilization of integral membrane proteins

Strategy	Variant generation	Read-out	Comments	Ref.
Alanine scanning mutagenesis	Systematic scanning mutagenesis Iterative recombination of stabilizing mutants	Individual screening for increased detergent stability	+ Well-established technique + Direct read-out of detergent stability – Very laborious approach – Restricted sequence permutations – No selection of synergizing mutations	[18–20]
Directed evolution for high expression	Error-prone PCR or: iterative saturation mutagenesis with combinatorial library of selected mutations	HT FACS-based selection of high-expressing variants	+ Fast HT selection approach + Increase of GPCR expression levels + Exploring large sequence space – Need for fluorescent ligand – Reliance on correlation between functional expression and stability – To date this method requires bacterial expression – Potential accumulation of non-stabilizing/neutral mutations	[45,46,48*]
Directed evolution for high stability (CHESS)	Error-prone PCR	HT FACS-based selection of variants with increased detergent stability	+ Fast HT-selection approach + Exploring large sequence space + Direct selection for parameters important for drug discovery (thermo-, detergent and long-term stability) – Need for fluorescent ligand – To date this method requires bacterial expression – Potential accumulation of non-stabilizing/neutral mutations	[49**]
Rational design	Consensus sequence design combined with computational prediction of metastable sites, followed by experimental verification of individual mutants	Individual screening for increased thermostability	+ Least laborious method + Ligand independent – Structural information needs to be available – Success rate may vary	[50**]

for detergent stability through evolution so that stabilizing mutations can be found relatively easily. However, some receptors will almost certainly be more difficult to be stabilized in this way and will require a larger sequence space to be sampled. Moreover, a mutation to alanine will mainly test for the *removal* of a deleterious side chain, but in general not introduce a new interaction. Even though alanine promotes helix formation most strongly in soluble proteins, there is no clear evidence that high helical propensity plays a decisive role in the stability of transmembrane helices [43].

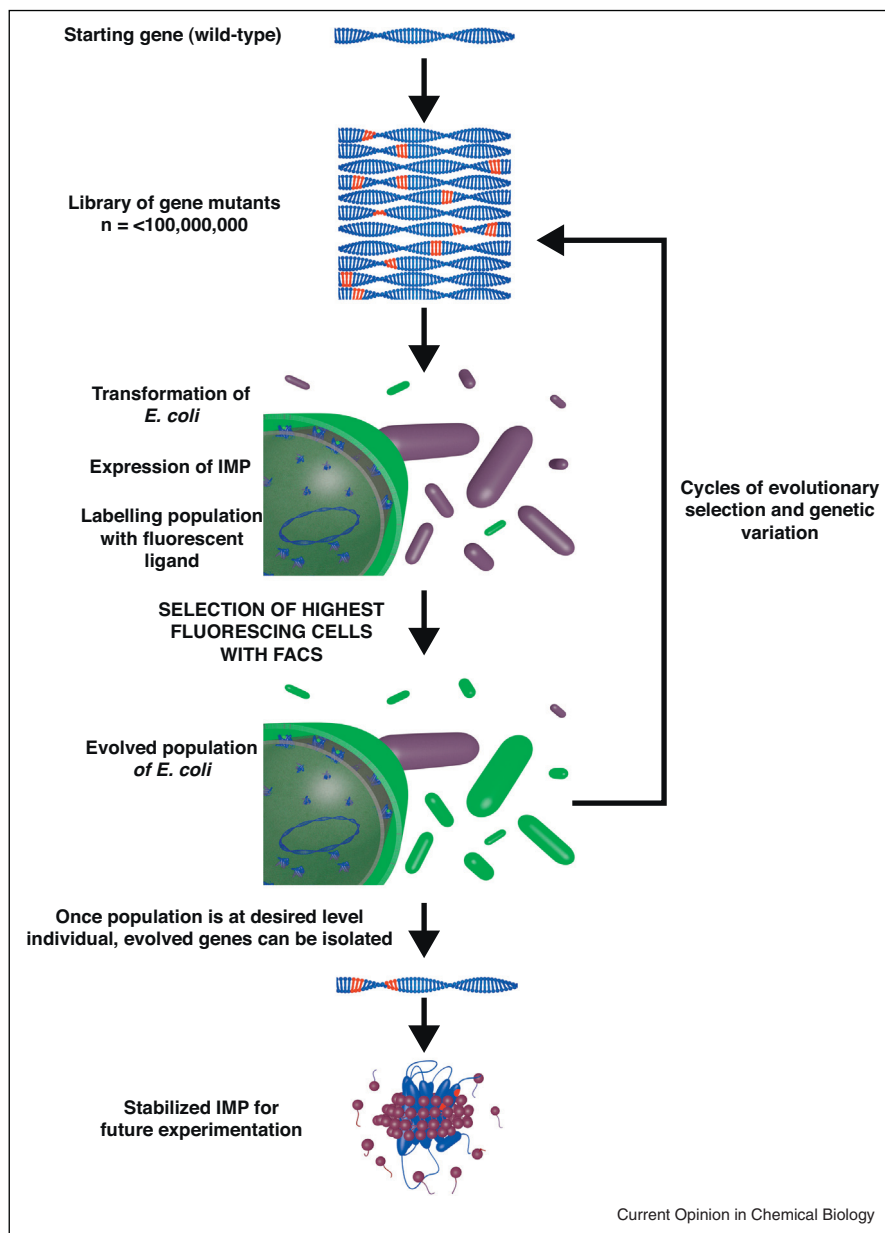
The seven transmembrane domains of a GPCR are typically over 300 amino acids long. A 300 amino acid stretch of protein has a theoretical sequence space of 20^{300} so that testing only single substitutions is actually a miniscule sample of the possible sequence permutations of the protein. Furthermore, because this technique is very laborious, only substitutions that improve the protein stability can be combined with each other to form the most stable variant. In natural evolution, mutations that by themselves have no effect on a protein can have a large effect when combined with others (neutral evolution). A strategy that can sample several orders of magnitude more

mutations than alanine scanning and especially select combinations of neutral mutations is directed evolution, which has also recently been applied to stabilize IMPs.

Directed evolution for high expression and stability

Directed evolution, which has been used for years to stabilize soluble proteins, allows for screening of a much larger number of mutants than conventional alanine scanning. It uses an iteration of diversification and selection and can optionally be used to focus on specific protein residues or regions. Accordingly, the iterative progression can cover a larger sequence space than contained in a single library, or covered by alanine scanning. Also, neutral mutations can occur, which can be the basis of additional positive selections in later rounds. Directed evolution has only recently been applied to improve the properties of IMPs. Considering that many IMP families are among the most evolutionary successful, their adaptability makes them perfect candidates for artificial, molecular evolution. Directed evolution typically involves creating a DNA library encoding millions of mutants of the protein of interest (Figure 3). Such a library can be made synthetically or with error-prone PCR or

Figure 3



Stabilizing IMPs through directed evolution. A gene library of the IMP of interest is generated, typically through random mutagenesis. *E. coli* is then transformed with the resultant library, generating a population of cells, each expressing a different IMP mutant. The population is treated with a fluorescent ligand, which binds to functional IMPs within the cells. Cells exhibiting the highest fluorescence (contain the highest amount of functional IMPs) are selected from the population using FACS, generating an evolved population. This population can be cycled through further rounds of mutagenesis and selection until the population has evolved to a high level of expression (high fluorescence). Individuals from this population can be isolated, their stability in detergent tested and the most stable, high expressing variants applied to downstream applications.

recombination. Mutants exhibiting the desired properties are then selected from the population. The key step is to use a selection assay that maintains a link between selected proteins and the genetic information encoding them so that the population can be adapted over generations of selection and eventually the identity of selected proteins determined by sequencing the corresponding

nucleic acid. For IMPs such as GPCRs, the main technical challenge was to find a selection system where millions of mutants can be produced in functional form, assayed and a genotype-to-phenotype link maintained.

The answer was to express the GPCR in the inner membrane of *Escherichia coli*, which had previously been

demonstrated to be possible with the right fusion proteins, although the expression level was low [44]. Because many changes that affect residue interactions in the protein may be generic and independent of the exact lipid or detergent environment, evolving a mammalian GPCR for high expression in the *E. coli* membrane is likely to result in a GPCR variant that is also more stable in detergent [45]. Another advantage was that transforming GPCR libraries into electrocompetent *E. coli* results in up to one hundred million cells expressing different GPCR library members. While screening one hundred million clones individually is not feasible, the use of a fluorescent ligand, which can specifically bind to folded GPCR proteins in the cells, and fluorescence-activated cell sorting (FACS), allowed the screening of up to one hundred million bacterial cells, each expressing a different GPCR mutant. Only cells exhibiting fluorescence levels in the top 1% of the population, that is expressing the highest levels of functional GPCR, were sorted and regrown for further generations of mutation and selection. This means that around six to seven orders of magnitude more mutants can be screened using this technique when compared to alanine scanning. Once the fluorescence of the sorted library population is significantly higher than the starting level, individual bacterial colonies can be isolated and the genetic identity of some of the evolved receptors determined. This bacterial display method has been used to engineer high expressing variants of several GPCRs [45,46] and a clear correlation between high expression and stability in some detergents is observed (determined by radioligand binding assays). Stability in the short-chain detergents needed for crystallization, however, required additional screening steps and new approaches for direct selection. Figure 3 depicts the general scheme of this directed evolutionary approach to IMP stabilization.

One approach that worked—and was very helpful in dissecting the structural features of a GPCR—was to utilize the bacterial display approach for selecting the highest expressing NTS₁ from libraries where every amino acid position was substituted for every possible amino acid [47•]. This saturation mutagenesis approach identified 30 positions where non-wild-type amino acids were preferred for higher functional receptor expression in the bacterial membrane. These mutations could then be recombined with each other into a new synthetic library, including three mutations from an alanine scanning study [20], and the corresponding wild-type residues at each position. Thereby, 2 highly diverse libraries were generated with theoretical diversities of $2^{33} = 8.6 \times 10^9$ mutants [48•]. Selecting the highest expressing mutants from this library identified 2 improved variants, called C7E02 and L5X, which both also exhibited stability in short chain detergents (determined by radioligand binding assays). To minimize the number of mutations of NTS₁, yet arrive at a GPCR stable in short-chain

detergents, the individual substitutions between C7E02 and D03 were individually tested and manually combined. One of the resultant mutants also showed very high stability in highly denaturing short-chain detergents and has been successfully crystallized and its structure solved in short-chain detergent, without the need for lysozyme insertion (Egloff *et al.*, unpublished data).

Cellular High Throughput Encapsulation, Solubilization and Screening (CHESS)

Bacterial display for high expression is able to easily select IMP GPCR variants with stability in longer chain detergents, however stability in harsher short-chain detergents required additional steps of receptor screening [48•]. The ability to perform directed evolution experiments where the stability of an IMP in detergent could be selected for *directly* would remove the need for such additional screening. Technically, this cannot be done using unmodified bacterial cells, because the detergent would solubilize the cells and thus remove the link between the IMP protein and the encoding gene. This problem was solved by encapsulating bacteria expressing GPCR mutants with alternating layers of polyelectrolytes, using a method called Cellular High throughput Encapsulation, Solubilization and Screening (CHESS) [49•]. This polymeric shell could allow the solubilization of the bacterial membranes from the inside out, without destroying the cells' size, shape and integrity. In short, the cell was converted into a semipermeable nano-container with solubilized contents. Importantly, the pore size of the encapsulating layer was small enough that solubilized GPCR proteins and encoding plasmids could not leak out of the capsule, and large enough that detergent molecules and fluorescently labeled ligands could diffuse inside the capsule. This enabled the selection of two different GPCRs using FACS, starting from highly diverse libraries, specifically for retaining function when solubilized in detergents. The resultant receptors were highly stable in short-chain detergents (determined by fluorescent ligand-binding assays), requiring no additional mutagenesis and screening after selection, and they crystallize readily in such detergents (Egloff *et al.*, unpublished data). Because the nano-containers are stable for weeks in detergents, CHESS enables the rapid screening of hundreds of millions of IMP mutants for detergent stability over long time periods (from hours to days) at elevated temperatures if desired (thermostability) and also for ligand preference.

At the moment, the limitations of the directed evolution techniques described above include the need for an IMP-specific fluorescent ligand to label cells or capsules containing functional protein (Table 1). Another is that to date these methods have used bacterial cells to express GPCRs, and not all IMPs are able to be expressed in *E. coli*, even though directed evolution can also be applied to alleviate this problem. It is technically feasible,

however, that these methods could be applied using eukaryotic cells, but this is yet to be proven. Another concern is the potential accumulation of non-stabilizing, neutral mutations through the evolutionary process. This, of course, could be controlled by systematically back-mutating the stabilized protein or, more elegantly, by recombining evolved populations of stable mutants with the wild-type sequence, followed by another round of evolutionary selection to enrich the most stabilizing mutations in the population.

Computational design of stabilized IMPs

While directed evolution is less laborious and allows the sampling of many more mutations compared to alanine scanning, there is still concern that mutational coverage may not be complete, at least at the stage of combining mutations. The ultimate way of stabilizing an IMP would be through knowledge-based design. However, we still know very little about how IMPs fold and remain stable in the membrane, nor do we understand the different environment in synthetic detergent micelles in atomic detail, such as the precise role of intercalation of detergent molecules in the protein, the access of solvent molecules, and general biophysical differences to a phospholipid bilayer. A step towards the design of stabilized IMPs was recently made by Chen *et al.* [50**]. This study took advantage of the known crystal structure of the $\beta 1$ -adrenoceptor [25], a GPCR, to computationally scan the protein and predict amino acids that may be destabilizing to the structure. Several positions were identified and mutations were designed to alleviate these metastable regions. These mutations were then tested experimentally and several engineered receptors were found to exhibit increased stability solubilized in mild, long-chain detergents (determined by radioligand binding assays). Unfortunately, no data were provided for the stability of these receptors in short-chain detergents [50**]. The disadvantage of this method is that a crystal structure is required to identify unstable regions of the protein, but probably the experimental data generated through the mutagenesis study of a number of IMPs with known structure will significantly increase the database for calibration, such that the combined experimental and computational engineering may hold much promise for the future.

Conclusions

Over the past few years IMP stabilization has grown to become an established field with proven utility in the biochemical and structural study of IMPs, particularly GPCRs. Significant innovation has provided the field with several possible methods for stabilizing an IMP of interest, each with advantages and disadvantages (Table 1). It is important to note that the functionality of stabilized IMP mutants needs to be tested thoroughly to assess the influence of mutations on function. This is particularly important for GPCRs, which are dynamic proteins that

interact with many ligands, depending on their conformation [51], and which subsequently interact with the trimeric G proteins, arrestins and other factors. It is likely that some stabilizing mutations in GPCRs impart conformational constraints on the protein, which may result in impaired signal transduction [30,48*]. Thus, the effect of stabilizing mutations should always be considered, depending on the desired application.

The challenge now is to apply these methods to different classes of IMPs so that we can learn more about the molecular aspects of their function. As more IMPs are stabilized we may start to learn more about the changes that are necessary for IMP stability, which will help future efforts to rationally or computationally design stable IMPs without the need for a crystal structure. Stabilized GPCRs have already provided us with high-resolution crystal structures that have been used for designing novel drugs [23,52–54]. The direct application of stabilized IMPs for drug discovery is an emerging field that may provide us with a new frontier for targeting these difficult proteins pharmaceutically.

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